DIETARY L-ARGININE AND ANTIOXIDANT VITAMINS E AND C INFLUENCE ON CARDIOVASCULAR PERFORMANCE IN CHICKENS

A Dissertation

by

JAIME BAUTISTA ORTEGA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2012

Major Subject: Poultry Science

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Approved by:

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ABSTRACT

Dietary L-Arginine and Antioxidant Vitamins E and C Influence on Cardiovascular Performance in Chickens. (May 2012)

Jaime Bautista Ortega, B.S., Universidad Autonoma Chapingo, M.Phil., University of Edinburgh, M.S., Oregon State University;

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Pulmonary hypertension syndrome (PHS) in broiler chickens adequately represents idiopathic pulmonary arterial hypertension (IPAH) in humans, a condition that affects 300 new patients each year in the US. The factors that trigger IPAH are poorly understood but an increase in reactive oxygen species in the circulation coincides with the onset of these conditions. Broiler chickens (n=583) were fed a control diet (CTL), containing 3,200 kcal of ME / kg of feed, 23% CP, 1.55% (wt / wt) Arginine (Arg) and 40 IU of VE (α-tochopherol) / kg of feed; a high-Arg diet (HA), CTL diet plus 0.8% (wt / wt) supplemental L-Arg HCl; or a high Arg and vitamin diet (AEC), the HA diet plus 200 IU α-tochopherol / kg of feed and 500 mg of ascorbic acid / L of drinking water 500 mg ascorbic acid / L of water (exp. 1 and 2) or Kg feed (exp. 3). Supplemented broilers were either exposed to hypobaric hypoxia or had a primary bronchus occluded (PBO) to induce PHS. Also, medial thickness was assessed in male broiler and Leghorn (n =80) chickens fed a CTL diet and subjected to pulmonary artery occlusion (PAO).

The results show that supplementation with Arg and VE plus VC have an additive effect on the velocity at which the pulmonary arterial pressure returned to basal levels in hypoxic chickens challenged with epinephrine. Also, supplementation increased xanthine oxidase (XO) activity in the vicinity of the pulmonary endothelium with no effect on NAD(P)H-oxidase activity or oxidative stress in hypoxic chickens subjected to

PBO. These enzymes are upregulated in humans with IPAH. Furthermore, supplementation reduced pulmonary artery reactivity to phenylephrine in hypoxemic broilers. Unsupplemented broiler chickens had a lower specific lung weight compared to unsupplemented Leghorns. Hypoxemic broilers showed thicker resistant pulmonary arteries and were more hypertensive than hypoxemic Leghorns. Leghorns were more hypoxemic and resistant to PHS than broilers. In conclusion, Arg and VE plus VC show an additive effect in the improvement of cardiovascular performance of hypoxemic broilers as well as in restoring reactivity to phenylephrine in hypoxemic pulmonary rings. Also, supplementation shows an additive effect in restoring XO activity in hypoxic broilers. Leghorns had a better ventilation capacity and better pulmonary vasodilation capacity than broiler chickens.

DEDICATION

To my wife and daughter:

Maria Luisa Flecha-Garcia

Sofia C. Bautista-Flecha

To my parents:

Juan Bautista-Hernandez and Eva Ortega-Hidalgo

Thank you for your support and motivation.

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Finally, thanks to my mother and father for their encouragement and to my wife for her patience and love.

TABLE OF CONTENTS

CHAPTER

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Pulmonary arterial hypertension syndrome (PHS) is a metabolic disorder that affects fast-growing broiler chickens. In susceptible broiler chickens, this disorder is initiated by an increase in either cardiac output (CO; e.g., because of increased metabolic rate) or pulmonary vascular resistance (PVR) to blood flow (e.g., exposure to hypobaric hypoxia) (Julian, 2007). Broiler chickens have been intensively selected for decades for fast growth and modern strains show a mismatch between oxygen demanding tissues (e.g. breast muscle) and oxygen supplying organs (e.g. lungs). Consequently, under commercial conditions susceptible individuals (3%-4% of the total placement) develop "functional hypoxemia" caused by a diffusion limitation of oxygen, a phenomenon that has been attributed to a high rate of blood flow in the pulmonary arteries (Wideman, 2001). Once hypoxemia ensues, the kidney secretes erythropoietin, which leads to an increased red blood cell count in turn causing an elevation in PVR, sustained pulmonary arterial pressure (PAP), ultimately leading to right ventricular failure (RVF).

Idiopathic pulmonary arterial hypertension (IPAH), also known as primary or essential pulmonary hypertension, has been defined as a sustained PAH of unknown origin, i.e. PAH is not secondary to any known cardiovascular anomaly (Copple et al., 2002). This condition affects 1-2 people per million in the general population and recent data from the Centers for Disease Control suggest that its incidence may be increasing. IPAH is characterized by a high PVR and a mean PAP ≥25 mm Hg at rest and ≥30 mm Hg during exercise. This disorder carries a high mortality, with the most common cause of death being RVF. Because this condition is generally diagnosed in advanced stages, i.e. when the patient shows overt clinical signs, the median survival is approximately 2.8 years.

It is still unknown what triggers IPAH and there is limited information regarding

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This dissertation follows the style of Poultry Science.

the early stages of its pathological progression. Efforts to elucidate the primary cause that lead to selective damage or alteration to the pulmonary vasculature has relayed on rodent models of PAH. However, most of these models do not adequately represent the human disorder. For example, monocrotaline-induced pulmonary vasculitis/hypertension causes systemic vessel endothelial damage and also involves an immunological response (Halliwell and Gutteridge, 1999). The mouse eNOS-/- model, endothelial nitric oxide synthase knock out, has the drawback that eNOS is expressed in all vascular beds and so it is not specific to study pulmonary disorders (Fagan et al., 2000). This model is also limited by the lack of vascular remodeling. The broiler chicken has been proposed as a model for IPAH and more recently it has been reported that the broiler chicken adequately represents human IPAH (Martinez-Lemus et al., 1999; Hamal et al., 2010; Alvarez-Medina et al., 2011). One of the advantages of the chicken model of IPAH is that it is tractable and the condition can be amplified (using a limited number of birds) using several methods including (a) exposure to low temperature (b) exposure to hypobaric hypoxia (c) unilateral pulmonary artery or extra-pulmonary bronchus occlusion (d) i.v. microparticle injection, and (e) chronic supplementation with thyroid hormone. Additionally, chickens and humans are monogastric and so they have a similar utilization of nutrients supporting a dietary approach to study IPAH.

The onset of PHS has been associated with an increased production of reactive oxygen species (**ROS**) and endothelial cell damage (Enkvetchakul et al. 1993; Wedgwood and Black, 2003; Pan et al., 2007; Nain et al., 2008a,b). One of the mechanisms proposed through which oxidative stress is implicated in the pathophysiological progression of PHS is by reducing availability of the NO through its reaction with superoxide to produce peroxynitrite, reducing smooth muscle vasodilation and contributing to a high PAP (Martinez-Lemus et al., 1999; Tan et al., 2007a).

In the present study, a set of experiments were carried out whereby broiler chickens were fed diets that potentiated the bioavailability of nitric oxide (NO) in the live chicken, which includes the cardiovascular system. This was achieved by feeding supplemental L-arginine (Arg; the essential amino acid that is the substrate of eNOS),

and so it was expected that the amount of NO produced in the broiler chicken would increase. Additionally, the experimental broiler chickens were further fed supplemented antioxidant vitamins, vitamin E (VE) and vitamin C (VC), which have been shown to protect NO against the action of reactive oxygen species (ROS); the latter readily convert NO to peroxynitrite thus losing NO's biological effects (Tan et al., 2007). Endothelium-derived NO maintains vascular tone because it signals the pulmonary artery smooth muscle to relax and thus in our experimental set up it is expected that NO will allow a ventilation-perfusion match within the lung of broiler chickens thus counteracting any imbalance between NO and endothelin-1 (vasoconstrictor) improving cardiovascular performance (Bowers et al., 2004). In another experiment, fast-growing broiler chickens were compared with Leghorn chickens (slow growing chickens; not susceptible to PHS) with regards to their physiological and lung morphological adaptations to an increased PVR caused by pulmonary artery occlusion in order to establish a connection between differential vascular remodeling of resistance arteries and related PHS-related parameters. The effect of supplemental Arg and VE and VC on ROS-producing enzymes xanthine oxidase and NAD(P)H oxidase were also investigated in relation to peroxynatrite formation at the expense of NO. Finally, the effects of supplemented Arg and VE and VC on the reactivity to phenylephrine were investigated in pulmonary artery rings using an in vitro approach. The overall goal of this research was to study the role of ROS in the early stages of PHS and to assess the physiological and morphological differences between broiler chickens and slow-growing Leghorn chickens that may explain their differential susceptibility to PHS.

CHAPTER II

LITERATURE REVIEW

IDIOPATHIC PULMONARY ARTERIAL HYPERTENSION IN HUMANS

Idiopathic pulmonary arterial hypertension (IPAH) (also known as primary or essential pulmonary hypertension) has been defined as a sustained PAH of unknown etiology, i.e. PAH is not secondary to any known cardiovascular anomaly (McLaughlin et al., 2009). This condition is characterized by a high pulmonary vascular resistance (PVR) and a mean pulmonary arterial pressure (PAP) ≥25 mm Hg at rest and ≥30 mm Hg during exercise. The increase in PVR has been attributed to large and intermediate-sized pulmonary arteries/arterioles in which there is intimal hyperplasia with medial and adventitial hypertrophy and hyperplasia. If left unattended, the disease carries a high mortality, with the most common cause being right ventricular failure (RVF). The incidence of the condition is approximately 6 cases per million people (McLaughlin et al., 2011). Recent data from the Centers for Disease Control suggest that the condition may be increasing. Women are twice more susceptible than men and the estimated median survival is approximately 2.8 years. The primary mechanism leading to endothelial dysfunction and ultimately to the formation of plexogenic arteriopathy, a defining lesion of idiopathic pulmonary hypertension (IPAH), remains to be identified (Simonneau et al., 2009; Tuder et al., 2009). In plexogenic arteriopathy, a tuft o capillary formation is present, producing a network that spans the lumens of small arteries.

RODENT MODELS OF IDIOPATHIC PULMONARY ARTERIAL HYPERTENSION

At present, the etiology of human IPAH is unclear and efforts to elucidate the primary causes that lead to selective damage to or alteration of the pulmonary

vasculature has relayed on rat models of PAH. The rodent models of human cardiopulmonary diseases, including PAH, have been reviewed (Stenmark et al., 2009). Selected models are described below.

Monocrotaline-induced pulmonary vasculitis/hypertension. Monocrotaline-induced vascular disease has been well-characterized in the rat and in other animal species. The proposed mechanism of injury includes selective pulmonary endothelial damage by the pyrrole metabolite of monocrotaline with induction of progressive pulmonary arteriolar muscularization. As blood flow through the lungs is increasingly restricted, pulmonary arterial hypertension develops. A single intra-peritoneal or sub-cutaneous injection (50-60 mg/kg body weight) of monocrotaline produces pulmonary hypertension in the rat starting two weeks post-treatment. Susceptible rats develop right ventricular hypertrophy and mortality starts at about one month. Rats that do not die within one or two months continue living with pulmonary hypertension for a long time. The drawback of this model is that monocrotaline also causes systemic vessel endothelial damage as proved by observed liver damaged in treated rats (Gomez-Arroyo et al., 2012).

Hypoxia-induced pulmonary hypertension**.** Hypobaric hypoxia and nitrogen dilution have been used to induce PAH in rats. A continuous three-week exposure of rats to a simulated altitude of 16,000 ft results in progressive pulmonary artery muscularization and PAH. Acute hypoxia induces PAH through pulmonary arteriolar constriction, which causes resistance to blood flow through the lung. Erythropoietin-mediated polycythemia follows with an increase of atrial natriuretic peptide levels in the blood. Exposure to hypoxia also stimulates production of vascular endothelial growth factor and expression of its receptors as well as expression of fibronectin and laminin, interleukin-1α, interleukin-6 and endothelin, all of which are linked to vascular remodeling. However, this model has been suggested not to reflect the whole cascade of the human condition.

Fawn Hooded Hypertensive rat (FHR). The inbred FHR is currently the only mammalian model of spontaneous PAH. The FHR shows sustained increases in PVR

correlated and has a genetic predisposition to develop extensive medial hypertrophy in pulmonary arterioles, but neointimal proliferation and complex occlusive lesions have not been reported in the FHR. This important feature distances the model from a true representation of human IPAH. The FHR also serves as an animal model of systemic hypertension, chronic renal failure and behavioral studies (e.g. response to serotonin drugs binding brain receptors).

Mice lacking endothelial nitric oxide synthase (eNOS-/-). The eNOS knockout mice model of hypertension was introduced in 1995. This model has allowed studying the importance of NO in both normal and abnormal murine pulmonary circulation. Since eNOS is expressed in all vascular beds it is not specific for pulmonary disorders. The reported lack of vascular remodeling presents another limitation of mouse models.

A CHICKEN MODEL OF IDIOPATHIC PULMONARY ARTERIAL HYPERTENSION

Under commercial conditions at sea level, 3% to 4% of the broiler chicken populations spontaneously develop idiopathic pulmonary arterial hypertension (IPAH; also known as pulmonary arterial hypertension syndrome (PHS) or ascites syndrome). There is agreement among the research community that PHS in broiler chickens can be precipitated by factors that increase the volume of blood circulating in the pulmonary vasculature or by those that increase the resistance to blood flow which ultimately alter the chicken's cardiovascular hemodynamics (Wideman, 2001) (Figure 1). Fast-growing broiler chickens have an increased pulmonary blood flow (cardiac output), generally believed to be caused by a fast-growth rate. Susceptible chickens then develop "functional hypoxemia" caused by a diffusion limitation associated with a high rate of blood flow in the pulmonary arteries (perfusion:diffusion mismatch). Once hypoxemia ensues, the kidney secretes erythropoietin which leads to an increase in red blood cell concentration thus further increasing vascular resistance. This vicious cycle leads to right ventricular hypertrophy and dilation and right atrio-ventricular valve insufficiency.

Affected broiler chickens die from right ventricular failure, suffocation (ascitic fluid accumulation in the abdominal cavity) or both.

Broiler chickens with IPAH show medial hypertrophy within 25 to 100 μm diameter inter-parabronchial pulmonary arterioles, which are homologous to the pre-acinar arteries of mammals (Xiang, et al., 2002; Moreno de Sandino and Hernandez, 2003; Tan et al., 2005a,b; Moreno de Sandino and Hernandez, 2006). Medial hypertrophy involves reduced apoptosis of smooth muscle cells in the pulmonary arterioles of broilers affected with IPAH compared with healthy-looking control (Tan et al., 2005). The chicken model is the only animal model of IPAH the develops plexiform lesions in pulmonary arteries (Wideman and Hamal, 2011; Wideman et al., 2011), a feature that they share with the human with IPAH.

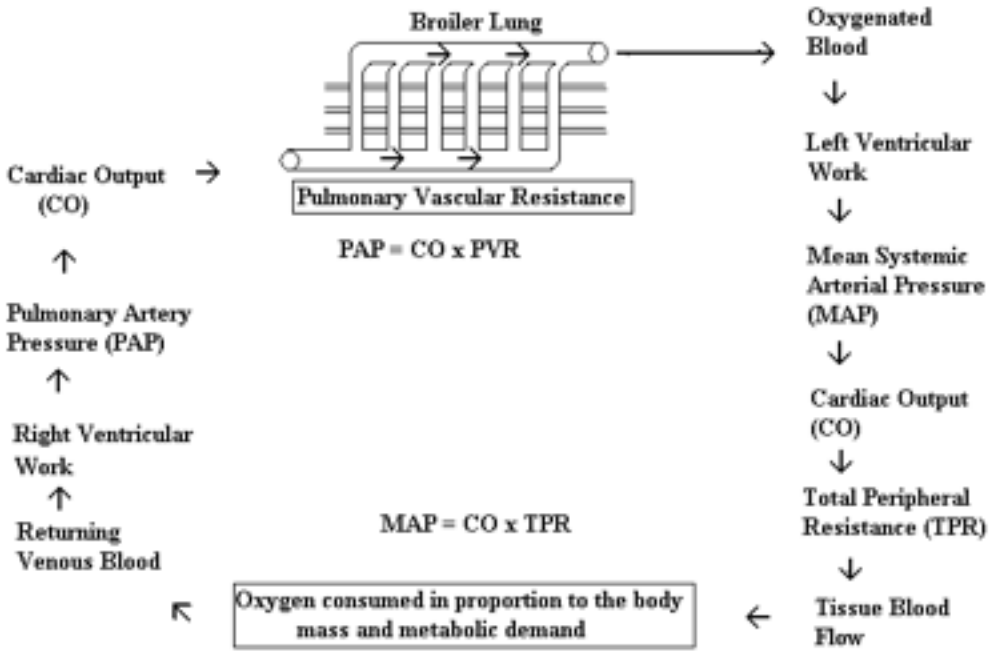

Figure 1. Schematic representation of the chicken's cardiovascular hemodynamics (adopted from (Wideman, 2001).

SELECTED METHODS TO AMPLIFY PULMONARY ARTERIAL HYPERTENSION

The following are methods that have been used by various investigators, and do not comprise all the available factors to artificially induce the condition.

Exposure to temperature below the thermo neutral zone (e.g. 16 °C) is a method that relies on the fact that any additional increment in the metabolic rate of susceptible broiler birds exposes the weakest link in the cascade of the pathophysiological progression of PAH. Any increment in the cardiac output (or blood flow through the lungs) will result in a more acute deployment of the "functional hypoxia" mentioned above thus exposing those birds that are at the borderline of the condition.

Exposure to chronic hypobaric hypoxia (simulated 10, 000 ft) is a method used because at high altitude mammals and chickens experience pulmonary arterial vasoconstriction in an effort to compensate for the differential partial O_2 pressure between the air (parabronchus) and blood capillaries. In the case of the broiler chicken, the already compromised pulmonary vasculature leads to pulmonary hypertension. Physiological responses to chronic hypoxia include erythropoiesis and angiogenesis to achieve reoxygenation of hypoxic tissue (Walshe and D'Amore, 2008). Moreover, chronic hypoxia can lead to adaptive responses such as vascular remodeling characterized by cell damage that results in hypertrophy and hyperplasia of the smooth muscle layer in the small arterioles of the lung (Tan et al., 2007b).

Unilateral pulmonary artery occlusion (PAO) is a surgical procedure that places a large workload on the right ventricle of the heart with the subsequent death of

susceptible individuals (Wideman and Kirby, 1995ab). By unilaterally occluding one pulmonary artery the blood volume perfusing the unobstructed lung (pulmonary artery) is doubled. Thus, a proportionally high cardiac output together with a low capacity non-compliant pulmonary vasculature can lead to a ventilation / perfusion mis-match hypoxaemia and to a rapid development of pulmonary hypertension in fast-growing broilers subjected to PAO (Koyama and Horimoto, 1983; Nagasaka et al., 1984; Reeves and Rubin, 1998). Wideman and Kirby (1995b) found that the response of the chicken's heart to induced pulmonary hypertension was very rapid and observed dilated hearts as early as 24 hours after PAO. In general, PAO initiates a progression of symptoms typical of those observed in broilers developing PHS spontaneously under a variety of environmental and commercial conditions or during exposure to experimental models such as cold or hypobaric hypoxia.

Intravenous microparticle injection. Wideman and Erf (2002) introduced the use of micro-particle injections in broiler chickens to occlude precapillary arterioles and trigger an increased pulmonary vascular resistance to blood flow leading to PHS. Cellulose microparticle suspension demonstrated to be most effective (Wideman et al., 2002). When male broilers were injected at 18 to 20 days of age with cellulose particles, survivor birds showed superior cardiopulmonary capacity when exposed to a moderate heat challenge (Wideman et al., 2003). The authors did not find any detrimental consequence due to the treatments such as respiratory alkalosis or chemoreceptor restrain on evaporating cooling. One of the potential side effects of injecting foreign particles into the circulation of birds is the eventual production of inflammatory response, as suggested by Wideman et al. (2002).

Supplemental triiodotyronine (T_3) to induce PHS in broiler chickens. Supplemental 0.5 mg T3/ Kg feed causes hypertension (assessed as cardiac hypertrophy) and ascitic accumulation in the abdominal cavity (clinical PHS) of treated broiler chickens. Studies led by Decuypere et al. (1994) demonstrated the link between thyroid function and PHS such that supplemental T_3 induced mortality in dose-dependent manner from 0.5 mg / Kg to 2 mg / Kg feed. This method is based on the fact that

thyroid hormone supplementation increases oxygen consumption and heat production (Wrutniak-Cabello et al., 2001) and thus increasing metabolic rate and pulmonary blood flow, both triggers of PHS.

In the present investigation PHS was induced using the following methods: (1) exposure to hypobaric hypoxia (2) primary bronchus occlusion (3) pulmonary artery occlusion. These PHS-inducing methods were chosen because of the availability of the expertise and materials needed to perform the surgical procedures and because of the limited number of birds were used.

ROLE OF NITRIC OXIDE IN VASCULAR HOMEOSTASIS

Nitric oxide (NO) has received considerable attention because research has shown its multiple positive effects. For example, NO is a potent vasodilator that can reduce pulmonary vascular resistance and ameliorate the development of pulmonary arterial hypertension (PAH) in humans and birds (Ramamurthi and Lewis, 1997; Chapman and Wideman, 2006a). Hepatocytes, macrophages, heterophils (avian equivalent to the mammalian neutrophil) and endothelial cells synthesize NO endogenously (Ramamurthi and Lewis, 1997; Chapman and Wideman, 2006a). Also, NO in the vascular system exerts control on blood pressure, inhibits platelet aggregation and killing of foreign organisms (Halliwell and Gutteridge, 1999).

Figure 2 shows the pathway and the elements that take part in the synthesis of NO in the endothelial cell in health and in disease (e.g. hypertension). Synthesis requires the presence of L-arginine, an electron donor of NAD(P)H, the cofactor tetrahydrobiopterin (BTH4) and endothelial nitric oxide synthase (eNOS).

Nitric oxide readily binds certain transition ions, and many of its physiological effects are exerted as a result of its initial binding to Fe^{2+} heme groups in the enzyme guanylate cyclase (Halliwell and Gutteridge, 1999).

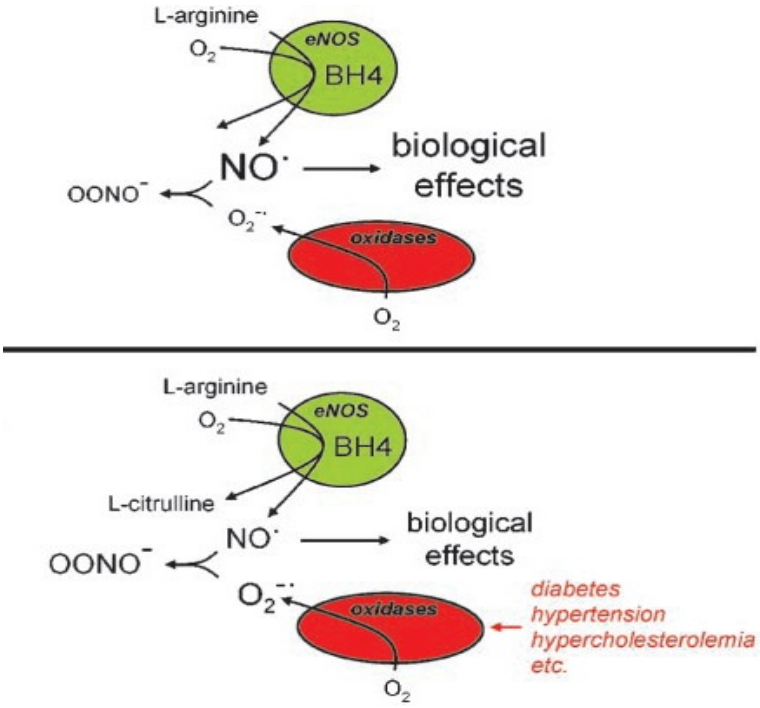

Figure 2. Schematic depicting the pathway of nitric oxide ($NO^{\cdot}$) production. Top panel, normal physiological conditions; bottom panel, disease conditions (Alp and Channon, 2004). BH4 = tetrahydrobiopterin; eNOS = endothelial nitric oxide synthase; $OONO^{-}$ = peroxynitrite.

Thus, NO synthesized by the vascular endothelial cells diffuses in all directions, but some of it reaches the underlying smooth muscle, bind to guanylate cyclase and activate it. As a result more cGMP is made, which lowers intracellular free Ca^{2+} and relaxes the muscle, dilating the vessels and lowering blood pressure.

Nitric oxide rapidly reacts ($k \approx 7 \times 109\ M^{-1}\ s^{-1}$) with superoxide to form peroxynitrite (Halliwell and Gutteridge, 1999).

Nitric oxide synthase exists in three iso-forms: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3). Although eNOS is constitutively expressed (house keeping gene) in the endothelial cell, eNOS gene transcription may be modulated by many factors.

CHAPTER III

L-ARGININE AND ANTIOXIDANT VITAMINS E AND C IMPROVE THE CARDIOVASCULAR PERFORMANCE OF BROILER CHICKENS GROWN UNDER CHRONIC HYPOBARIC HYPOXIA*

INTRODUCTION

Pulmonary hypertension syndrome (PHS) is a metabolic disorder that affects fast-growing broiler chickens. In susceptible broiler chickens this disorder is initiated by an increase in either cardiac output (CO) (e.g. because of increased metabolic rate) or vascular resistance to blood flow (e.g. exposure to hypobaric hypoxia) (Julian, 2007). When broilers are raised at high altitude the incidence of the condition increases because environmental hypoxia at high altitudes causes vasoconstriction in the pulmonary vasculature increasing vascular resistance and pulmonary arterial pressure (PAP) (Burton et al., 1968; Julian, 2007). Physiological responses to chronic hypoxia also include erythropoiesis and angiogenesis to achieve re-oxygenation of hypoxic tissue (Walshe and D'Amore, 2008). Moreover, chronic hypoxia can lead to adaptive responses such as vascular remodeling characterized by cell damage that results in hypertrophy and hyperplasia of the smooth muscle layer in the small arterioles of the lung (Tan et al., 2007b).

L-arginine (Arg) is an essential amino acid in the chicken and it has been suggested that its dietary inclusion at the levels recommended for maximal growth rate do not agree with those required for maximal nitric oxide (NO) production (Dietert and Austic, 1994). L-arginine is the substrate of endothelial nitric oxide synthase (eNOS), an enzyme that synthesizes NO, a potent vasodilator (Dudzinski and Michel, 2007) and

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* Reprinted with permission from "L-Arginine and antioxidant vitamins E and C improve the cardiovascular performance of broiler chickens grown under chronic hypobaric hypoxia" by Bautista-Ortega, J and C.A. Ruiz-Feria, 2010. Poult. Sci., 89, 2141-2146, Copyright 2010 by Poultry Science Association Inc.

anti-mitogenic (Tan et al., 2005). Basal levels of endothelium-derived NO signals the pulmonary artery smooth muscle cells to relax thus maintaining vascular tone (Govers and Rabelink, 2001). Supplemental L-arginine improves cardiovascular performance in birds exposed to cold environments (Lorenzoni and Ruiz-Feria, 2006; Ruiz-Feria, 2009) and has been reported to reduce PHS in broiler chickens (Wideman et al., 1995; Tan et al., 2007b), although the results have not been consistent.

Vitamins E (VE) and vitamin C (VC) are important cellular antioxidants in the animal system, including chickens (Serbecic and Beutelspacher, 2005; Surai, 2006). Vitamin E is a cell membrane antioxidant that protects cell integrity by reducing polyunsaturated fatty acid oxidation while VC is a cytosolic antioxidant that restores the antioxidant capability of oxidized VE (Guney et al., 2007). The onset of PHS has been associated with an increased production of reactive oxygen species (ROS) and endothelial cell damage (Enkvetchakul et al. 1993; Wedgwood and Black, 2003; Pan et al., 2007; Nain et al., 2008a,b). The involvement of ROS in the etiology of PHS has prompted the study of the combined effect of Arg and antioxidant vitamins (VE and VC) on cardiovascular performance in broilers grown under cold temperature; we previously found that at high levels of VE (400 IU / kg of feed) the positive effects of Arg were negated, possibly due to the pro-oxidative effects of VE (Lorenzoni and Ruiz-Feria, 2006). In another series of experiments we found that with lower levels of VE (200 IU / kg of feed) the combined effect of Arg and VE improved cardiopulmonary response after an acute challenge with epinephrine compared with Arg alone (Ruiz-Feria, 2009). In the same work we found that birds fed a combination of Arg, VE and VC had a better cardiopulmonary response than birds fed either a combination of Arg and VE or a combination of Arg and VC. The amplification of PHS through cold stress is different from that caused by hypobaric hypoxia; in the first case, PAP is primarily increased by an enhanced CO in response to an increase in metabolic demand, while in the second case the increase in PAP is caused primarily by pulmonary vascular constriction increasing pulmonary vascular resistance. There is no information on the combined effect of Arg and antioxidant VE and VC on the development of PHS in broiler chickens

raised under hypobaric hypoxia. In this study we evaluated the effects of Arg and a combination of Arg with antioxidant vitamins, VE and VC, on cardiopulmonary pulmonary function and parameters associated with PHS in broilers raised under hypobaric hypoxia. It is hypothesized that Arg and antioxidant vitamins may play complementary or synergistic roles to increase NO bioavailability and reduce oxidative stress damage, thus improving cardiopulmonary performance.

MATERIALS AND METHODS

Two hundred, one-day-old Cobb 500 broiler chicks were used to assess the effect of dietary Arg, VE, and VC on cardiopulmonary performance and parameters related with PHS. The chicks were wing-banded and randomly allocated to one of three dietary treatments: a control diet (CTL), containing 3,200 kcal of ME / kg of feed, 23% CP, 1.55% (wt / wt) Arg and 40 IU of VE (α-tochopherol) / kg of feed; a high-Arg diet (HA), CTL diet plus 0.8% (wt / wt) supplemental L-arginine HCl (SAFC Supply Solutions, St. Louis MO); or a high Arg and vitamin diet (AEC), the HA diet plus 200 IU α-tochopherol / kg of feed (Producers Cooperative Association, Bryan, TX) and 500 mg of ascorbic acid / L of drinking water (Sigma-Aldrich Corp., St. Louis, MO). The diets were iso-caloric and iso-nitrogenous and formulated to meet or exceed all of the NRC (1994) requirements. Supplemented vitamin C in drinking water was prepared daily. The chicks were brooded conventionally with temperature starting at 32° C and decreasing 2° C each week, under a constant lighting program. The chicks were housed in six wire cages (56 cm long X 33 cm wide X 28 cm high) under normoxic conditions for 4 (Batch 1), 7 (Batch 2) or 10 days (Batch 3) and then the cages were placed in two plastic cylindrical hypobaric chambers (185 cm long and 58 cm diameter) in which the chickens were exposed to a simulated altitude of 3,000 m (10,000 ft) above sea level (14.5 kPa of partial pressure of O_2) to amplify the incidence of PHS. The desired simulated altitude (low partial pressure of O_2) was achieved by pulling a vacuum through the chamber with a Roots Universal blower (Dresser industries, Connersville, IN). The blower was

reversed to pull air through the air plenum. The chicks were kept inside the hypobaric chambers continuously except for 40 min to clean and re-feed the chicks, 15 min to decompress the chambers and 15 min to reach the desired vacuum level. In birds from Batch 3 the chambers were kept open from d 30 onwards while conducting the cardiopulmonary performance tests. The experimental chambers were monitored daily for mortality and the heart was dissected to determine right ventricle weight/ total ventricle weight ratio (RVW/TVW) (Burton et al., 1968) in all birds. Hematocrit (Hc) was measured weekly (d 7 to 28, Batch 1 and 2), whereas heart rate (HR; beats/min) and ECG s-wave amplitude (S-Wave) were recorded at d 14 and d 21 (Batch 2). From d 28 to 42, clinically healthy birds were selected for cardiovascular performance (Batch 3, n = 7 to 12 / treatment) as described earlier (Ruiz-Feria, 2009). Briefly, a surgical plane of anesthesia was induced with intramuscular injections of allobarbital (Dial mixture, 5, 5-diallyl-barbituric acid; 25 mg/kg of BW i.m., Sigma-Aldrich, St. Louis, MO) with lidocaine hydrochloride (2% s.c., xylocaine, Astra-Zeneca, Wilmington, DE) injected as supplemental local anesthetic at incision sites. The birds were fastened in dorsal recumbency on a surgical board while maintaining the temperature at 30 °C. The left brachial artery was isolated and cannulated with 30 cm of heparinized polyethylene tubing (PE-50 polyethylene, Solomon Scientific, San Antonio, TX). The left brachial vein was cannulated using 30 cm of heparinized Silastic tubing (0.012 in internal diameter x 0.025 in outside diameter; Dow Corning Corporation, Midland, MI) and the proximal end was advanced through the vein and right ventricle until it reached the pulmonary artery. The distal end of both the PE-50 polyethylene and Silastic tubing were attached to blood pressure transducers interfaced with a PowerLab data acquisition system (ADInstruments Pty Ltd, Bella Vista, Australia) for the continuous measurement of mean systemic pressure (MAP, mm Hg), PAP (mm Hg), and HR. After surgical preparation was completed and a 10 min stabilization period has elapsed, control data was recorded for 10 min. During this period PAP and MAP readings were taken at 180, 120, and 60 s before an epinephrine (Epi; 4-(1-hydroxy-2-[methylamino]ethyl)-1,2-benzenediol hydrochloride, Sigma) challenge (0.5 mg / kg BW) to obtain basal values.

The vasodilation capacity of the pulmonary arteries was estimated by measuring the time that birds within each dietary treatment took to return to PAP levels before the Epi challenge. The PAP, MAP, and HR responses were measured at 30, 60, 120, 180, 300, 600, and 1,200 s after the Epi challenge. After the first 1,200-s recovery period, a second Epi challenge was applied and the same points described for the first challenge were used to sample the resulting curve. A typical recording generated by the physiograph used, showing continuous PAP and MAP values, is presented in Figure 3. Heart rate was obtained by counting systolic peaks over time coincident with each sampling time interval. At the end of the experiment birds were humanly killed and the RVW/TVW was determined.

All experimental protocols were approved by the institutional animal care committee at Texas A&M University. Hematocrit and RVW/TVW, and values for PAP, MAP and HR within sampling points, were analyzed using a one-way ANOVA and means were separated by the Student-Newman-Keuls method. The PAP, MAP and HR values were analyzed within group over time using the repeated measures ANOVA (Jandel Scientific, 1994) and means were separated by the Student-Newman-Keuls method. Samples -180, -120, and -60 were considered baseline values for the first Epi challenge, and the sampling time of 1,200 s was considered as baseline value for the second Epi challenge. Differences were declared when the PAP, MAP, and HR means during the Epi challenges were statistically different ($P < 0.05$) from all respective baseline values.

RESULTS AND DISCUSSION

Chronic hypoxia results in the development of chronic pulmonary hypertension and pulmonary vascular remodeling (Stenmark et al., 2005). The effects of supplemental Arg in combination with antioxidant vitamins (VE and VC) have not been investigated in broiler chickens exposed to chronic hypobaric hypoxia.

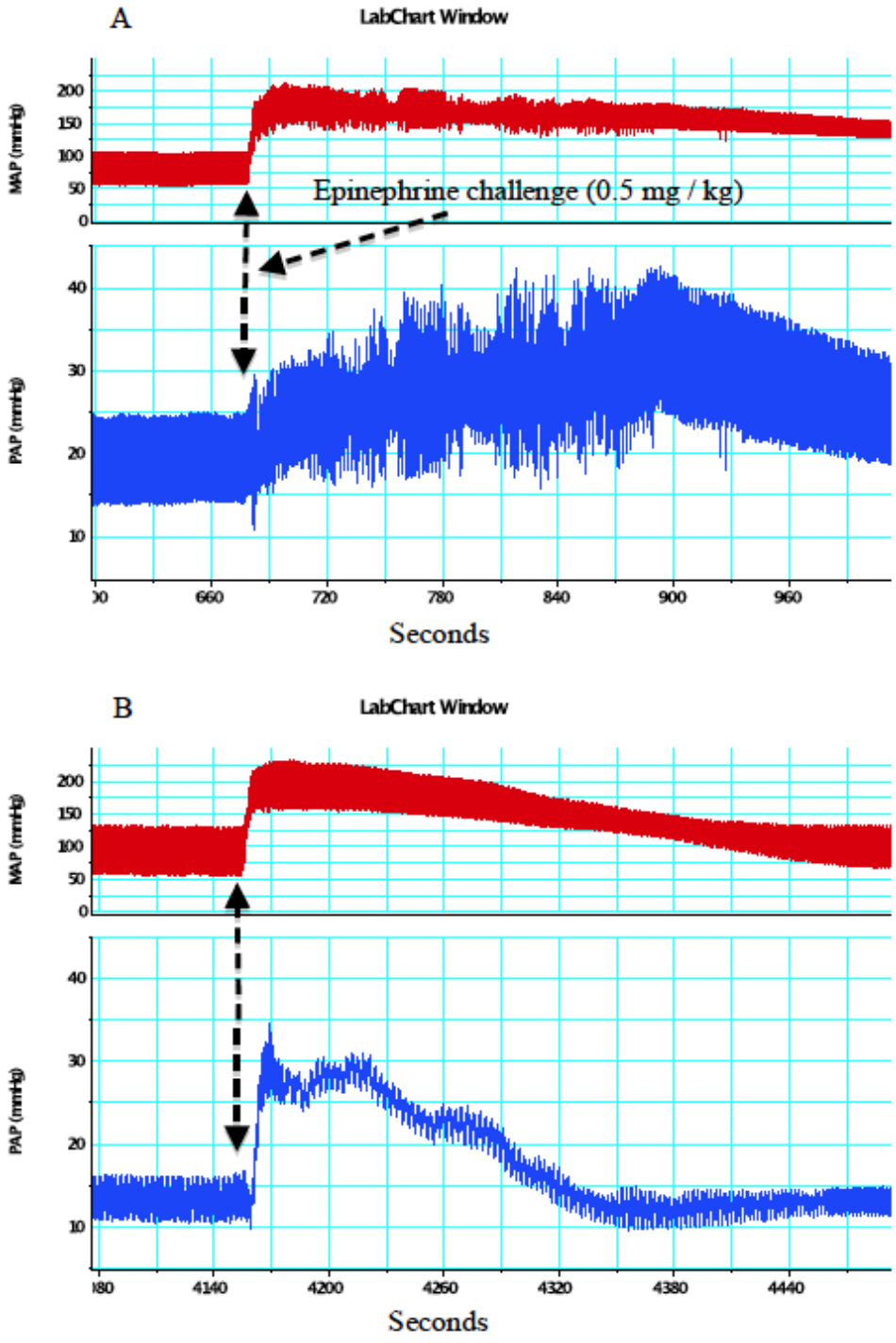

Figure 3. Sample physiograph recordings from 2 individual birds. (A) High-Arg group and (B) control group, in batch 3, showing continuous values for mean arterial pressure (MAP) and pulmonary arterial pressure (PAP) after epinephrine challenge.

The present study was conducted to assess the effect of supplemental Arg and antioxidant VE and VC on cardiovascular performance of broiler chickens chronically exposed to a simulated altitude of 3,000 m above sea level. It is our hypothesis that supplemental Arg will serve as substrate for enhanced production of NO whereas the antioxidant VE and VC may protect NO from inactivation by free radicals, showing additive or synergistic effects in maintaining NO bioavailability and improving cardiopulmonary performance under hypoxic conditions.

We did not find differences in the basal PAP values among chickens in the different dietary treatments (sampling points -180, -120 and -60; Table 1), perhaps because we selected only clinically-healthy chickens and presumably the most resistant individuals in the group; for instance, in broiler chicken populations there are a few individuals showing high resistance to PHS when exposed to cold temperature (Wideman and French, 1999).

The PAP increased within 30 s after the first Epi challenge in all treatment groups ($P < 0.05$; sampling point 30 vs. basal PAP values). The PAP remained high and was not different among treatments at 30 (peak PAP values) and 60 s after challenge. Looking at the PAP performance within groups, it took 180 s after the Epi challenge for chickens in the CTL group to return to their own basal PAP values, whereas chickens in the HA and AEC groups returned to their respective basal PAP values in only 120 s. However, there were not differences in PAP among treatments at any of the sampling periods. Thus, considering the time required for the PAP to return to basal PAP values, the best vasodilatory response following the first Epi challenge was achieved by chickens in the HA and AEC groups.

These results showed the beneficial effect of Arg on cardiovascular performance of birds exposed to hypobaric hypoxia, whereas the added effect of the antioxidant vitamins (VE and VC) was not apparent at this time. We have previously reported that dietary Arg correlates with Arg concentration in the plasma of broiler chickens (Ruiz-Feria et al., 2001).

Table 1. Pulmonary arterial pressure (PAP, mmHg) and mean arterial pressure (MAP, mmHg) after 2 epinephrine challenges (Epi; 0.5 mg / kg of body weight at time 0). Broiler chickens were fed a basal corn-soy meal-based diet (CTL) or the basal diet supplemented with arginine (HA), or a combination of arginine, vitamin E, and vitamin C (AEC) and grown under hypobaric conditions.

		PAP (mean ± SE, (N))			MAP (mean ± SE)		
Epi	Time, s	CTL (12)[1]	HA (7)	AEC (10)	CTL (12)	HA (7)	AEC (10)
	-180	20.2 ± 0.9	20.2 ± 1.2	19.3 ± 1.0	80.7 ± 6.2	95.2 ± 8.0	92.4 ± 7.8
	-120	20.1 ± 0.8	19.7 ± 1.1	19.3 ± 0.8	88.1 ± 6.2	97.2 ± 8.0	94.0 ± 7.8
	-60	20.2 ± 0.7	19.3 ± 1.0	19.5 ± 0.8	80.4 ± 6.2	100.8 ± 8.0	95.4 ± 7.8
1st	0						
	30	29.6 ± 1.6 *	29.6 ± 2.1*	30.5 ± 1.7 *	176.2 ± 6 2*	195.2 ± 8.0*	174.5 ± 7.8*
	60	27.5 ± 1.7 *	26.7 ± 2.3*	26.6 ± 1.9 *	160.1 ± 6.2*	180.9 ± 8.0*	158.7 ± 7.8*
	120	24.5 ± 1.9 *	25.2 ± 2.6	24.2 ± 2.2	137.4 ± 6.2*	151.6 ± 8.7*	147.7 ± 7.8*
	180	22.8 ± 1.8	24.0 ± 2.6	23.3 ± 1.8	126.6 ± 6.2*	144.2 ± 8.7*	141.0 ± 7.8*
	300	21.8 ± 1.5	23.6 ± 2.2	23.0 ± 1.7	102.1 ± 6.2*	113.0 ± 8.0	110.3 ± 7.8
	600	20.6 ± 1.4	18.6 ± 1.9	22.1 ± 1.3	91.5 ± 6.2	102.5 ± 8.0	91.8 ± 7.8
	1,200	18.5 ± 1.2	18.3 ± 1.6	21.5 ± 1.2	89.4 ± 9.9	93.7 ± 11.9	104.9 ±7.9
2nd	0						
	30	32.6 ± 1.8*[a]	26.2 ± 2.5*[b]	33.5 ± 1.8*[a]	167.4 ± 9.9*	162.1 ± 11.9*	164.6 ± 8.2*
	60	29.1 ± 1.6*[a]	22.9 ± 2.1*[b]	29.5 ± 1.6*[a]	159.7 ± 9.9*	153.6 ± 11.9*	157.9 ± 8.2*
	120	25.0 ± 1.4*	21.9 ± 1.9*	24.0 ± 1.5	144.2 ± 9.9*	140.3 ± 11.9*	138.2 ± 8.2*
	180	22.4 ± 1.3*	20.7 ± 1.8*	22.5 ± 1.3	128.2 ± 9.9*	130.8 ± 11.9*	123.0 ± 8.2
	300	22.0 ± 1.2*	20.7 ± 1.7	20.2 ± 1.3	110.5 ± 9.9	120.3 ± 12.8	103.7 ± 8.2
	600	20.5 ± 1.2	19.8 ± 1.0	17.6 ± 1.0	85.2 ± 6.2	106.1 ± 8.9	95.1 ± 3.0
	1,200	19.5 ± 1.0	19.3 ± 1.4	17.9 ± 1.1	86.2 ± 10.5	95.1 ± 12.8	90.9 ± 8.2

[1]CTL, basal diet containing 3,200 kcal of ME / kg of feed, 23% CP, 1.55% (wt / wt) Arg and 40 IU of VE / kg of feed; HA, a high-Arg diet, CTL diet plus 0.8% (wt / wt) supplemental L-arginine HCl; AEC, high Arg and antioxidant vitamin diet (AEC), the HA diet plus 200 IU α-tochopherol / kg of feed and 500 mg of ascorbic acid / L of drinking water. [a,b] Means within a row, and within the same parameter, lacking a common superscript differ ($P \leq 0.05$). [*]Means with an asterisk are different from its respective basal value (time -180, -120, and -60 for the first challenge, and time 1,200 for the second challenge; $P \leq 0.05$

Therefore, it is possible that Arg was capable of providing the substrate for eNOS to produce sufficient NO to improve the pulmonary vasorelaxation capacity after the first Epi challenge, as reported elsewhere (Lorenzoni and Ruiz-Feria, 2006; Ruiz-Feria, 2009). Following the second Epi challenge the PAP increased within 30 s in all treatment groups compared with their respective basal PAP and remained high in all treatment groups up to 60 s after the challenge. Also at this time (30 and 60 s after challenge) birds in the HA group had lower ($P < 0.05$) PAP than birds in the CTL and the AEC groups (Table 1). At both, 120 and 180 s after the second Epi challenge, there were no differences in PAP values among treatments; however, at this time the AEC group had returned to its basal PAP value, whereas birds in the CTL and HA still had PAP values higher than that of their respective basal PAP levels (time 1,200). After 300 s of the second Epi challenge, the PAP in the CTL group was still higher compared to its basal PAP values, whereas chickens in the HA group had returned to its basal PAP values. Therefore it took 60, 180, and 300 s for the AEC, HA, and CTL groups, respectively, to return to their respective pre-challenge PAP values. Thus the best vasodilatory response after the second Epi challenge was achieved by the AEC group, which showed the fastest return to the PAP basal values. Also, the HA birds had a better pulmonary vasodilation response than that of the CTL broilers because in the former it took a shorter time for the PAP to reach the basal PAP levels. In addition, the fact that 30 s and 60 s after challenge the HA birds had lower PAP values than birds in the CTL and the AEC groups provided further evidence that the HA birds had a better pulmonary vascular performance. These results suggest an additive effect between Arg and the antioxidant vitamins E and C on pulmonary vasodilation. Most likely, after the first Epi challenge, birds in the CTL group used the available Arg in their system to produce NO whereas birds in the Arg-supplemented groups the supplemental Arg provided an extra source of substrate for eNOS to produce higher amounts of NO, improving pulmonary vasodilatation. Furthermore, birds from the AEC group had the added benefit that the antioxidant vitamins provide, suggesting that the available NO was protected from ROS, increasing its biological activity resulting in a better vasodilatory response after

challenge. Oxidative stress due to increased production of ROS has been implicated in the pathophysiology of pulmonary hypertension (Enkvetchakul et al., 1993; Bowers et al., 2004; Nain et al., 2008a). Superoxide and NO react very rapidly at a rate (third order reaction) estimated to be 6.7 X 10^9 M^{-1} s^{-1} to form peroxynitrite (Goldstein and Czapski, 1995). This reaction is approximately three times faster than the dismutation of superoxide by superoxide dismutases, thus an increased generation of superoxide in the vascular wall may inhibit the physiological functions of NO (Kodja and Harrison, 1999). Alternatively, NO bioactivity can be impaired in the presence of peroxides, such as hydrogen peroxide (Thomas et al., 2006), which has been associated with PHS (Nain et al., 2008a) and vascular disease (Thomas et al., 2006). These authors proposed that hydrogen peroxide exerts its detrimental effects by promoting oxidation of synthesized NO; these oxidation products can lead to blood vessel damage and potentially exacerbate vasoconstriction. In a healthy system VE and VC neutralize ROS (Atkinson et al., 2008). Vitamin E maintains pulmonary endothelial cell membrane integrity by stopping lipid peroxidation of lipoproteins and so it acts as a defense against oxidative stress (Clarke et al., 2008). Vitamin C restores VE (Liu and Lee, 1998; Serbecic and Bautelspacher, 2005) most likely by recycling the tocopheroxyl radical (Bisby and Parker, 1995; Nagaoka et al., 2007). Also, ascorbate may enhance endothelium-derived vasodilation by scavenging superoxide radicals which inactivate NO as well as increase the production of peroxynitrite (Dudgeon et al., 1998). Therefore, in the present experiment, supplemental antioxidant vitamins, VE and VC, may have acted in a cooperative way to protect NO and extend its bioavailability (Serbecic and Bautelspacher, 2005). It is also possible that the antioxidants may have also protected the endothelium thus maintained a normal NO bioavailability in Arg and antioxidant supplemented birds (Rossig et al., 2001; Thomas et al., 2006; Pan et al., 2007). In addition, NO has been shown to act in a cooperative manner with α-tocopherol to inhibit lipid peroxidation processes thus potentially protecting α-tocopherol from oxidation (Rubbo et al., 2000).

The PAP response pattern seen in the AEC broilers following an Epi challenge differs from those of our previous reports (Ruiz-Feria, 2009), in which the peak PAP after the

Epi challenge (30 and 60 s) was lower in birds fed an AEC diet compared to that of birds fed a CTL diet, whereas in this experiment the PAP after the Epi challenge was not different between the AEC and the CTL group. These differences may be due to the different mechanism associated with the progression leading to terminal pulmonary hypertension. In this experiment we used hypobaric hypoxia, while in previous experiments we used low temperature exposure (Ruiz-Feria et al., 2001; Lorenzoni and Ruiz-Feria, 2006; Ruiz-Feria 2009); the first involves increases in pulmonary vascular resistance, while the second involves increases in cardiac output.

The response pattern of MAP to the Epi challenges mimicked that of the PAP (Table 1). MAP increased sharply within 30 s after the two Epi challenges. In birds from the CTL group the MAP remained high 300 s following the first Epi challenged whereas in the HA and CTL birds the MAP remained high for 180 s. Following the second Epi challenge, the MAP remained high for 180 s in the CTL and HA birds whereas in the AEC birds MAP remained high for 120 s. There were no significant differences among treatments in MAP at any of the sampling points analyzed suggesting that the differences in PAP described earlier were due to differences in pulmonary tone.

There were no differences among treatments in Hc values at any of the ages investigated in broiler chickens from Batches 1 and 2. Also, in broiler chickens from Batch 1, there were no differences in HR between treatment groups at wk 2 and 3. In addition, at the same ages there were no differences in the S-wave amplitude between broilers from the different experimental groups (Data not shown).

Supplemental Arg and antioxidant VE and VC did not reduce the incidence of PHS in hypoxic broilers most likely because exposure to simulated 3,000 m (10,000 ft) altitude was very severe. In the first Batch of chickens the hypoxia challenge lasted from d 4 to d 28 and there were no significant differences among treatments in PHS incidence: 35%, 35% and 29% for the CTL, HA and ACE group of broilers, respectively. In the second Batch of birds the hypoxia challenge lasted from d 7 to d 40 and the PHS incidence was 9%, 13% and 9% for the CTL, HA and AEC birds, respectively. In the third Batch the hypoxia challenge lasted from d 10 to d 30 and only one case of PHS was

observed in the CTL group. Differences in PHS incidence were influenced by the age at which hypobaric hypoxia challenge started as well as by the duration of the exposure. The RVW/TVW ratio was not different among treatments in any of the Batches.

In conclusion, supplemental Arg improved the pulmonary vascular performance of hypoxic broiler chickens and its effects were further improved by the addition of the antioxidant VE and VC. Arg and antioxidant vitamins may have played complementary or synergistic roles to increase NO bioavailability and reduce oxidative stress damage, thus improving cardiopulmonary performance. However, these supplemented levels of Arg, VE and VC did not prevent the development of PHS. It seems as if the exposure of broilers to simulated 3,000 m above sea level was too severe and overrode any beneficial effects of the experimental diets. Further research is warranted to quantify oxidative stress lesions or metabolites in pulmonary arterial beds of chickens supplemented Arg and antioxidant vitamins.

CHAPTER IV

SUPPLEMENTAL L-ARGININE AND ANTIOXIDANT VITAMINS E AND C RESTORE XANTHINE OXIDASE ACTIVITY DEPRESSED BY HYPOBARIC HYPOXIA WITHOUT AFFECTING NAD(P)H-OXIDASE ACTIVITY OR OXIDATIVE STRESS*

INTRODUCTION

The onset of pulmonary hypertension syndrome (PHS) in broiler chickens has been associated with an increased production of reactive oxygen species (ROS) and endothelial cell damage (Enkvetchakul et al., 1993; Wedgwood and Black, 2003; Pan et al., 2007; Nain et al., 2008a,b). Sources of ROS related to pulmonary hypertension include uncoupled eNOS (Sud et al., 2007), oxidases such as xanthine oxidase (XO) and NAD(P)H oxidase (NOX) (Zalba et al., 2000; Jankov et al., 2008; Warwick et al., 2008; Guzik and Griendling, 2009), and uncoupled mitochondria (Maxwell et al., 1996).

NOX has been described as the only known enzyme whose sole purpose is to produce ROS in various species and tissues, and is often associated with pathological conditions of the vasculature, including pulmonary hypertension (Selemidis et al., 2008; Lassegue and Griendling, 2010). The role of NOX on PHS in broiler chickens has not been studied. On the other hand, increased levels of uric acid, attributed to an increased XO activity, have been reported in humans with PH (Warwick et al., 2008). Poss et al., (1996) provided evidence that cell-bound XO can impair vascular cell function by inhibiting nitric oxide (NO)-dependent signal transduction. Although XO has been reported to be undetectable in the lung of healthy broilers (Carro et al., 2009), it has been

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shown to be attached to red blood cell membranes (Al-Khalidi and Chaglassian, 1965), and is therefore is in close contact with the pulmonary vascular endothelium.

L-arginine is an essential amino acid for birds and is the substrate of endothelial nitric oxide synthase (eNOS), the enzyme that synthesizes NO, a potent vasodilator (Dudzinski and Michel, 2007). eNOS is constitutively expressed and basal levels of NO enable the pulmonary vascular smooth muscle to maintain vascular tone (Govers and Rabelink, 2001). Supplemental Arg has been shown to improve cardiovascular performance in broiler chickens exposed to temperatures below their thermoneutral range (Lorenzoni and Ruiz-Feria, 2006; Ruiz-Feria, 2009) and has been reported to reduce PHS incidence (Wideman, et al., 1995; Tan et al., 2007b), although the results have not been consistent. In this regard, Ruiz-Feria et al. (2001) found no effect of supplemental Arg on PHS incidence in broilers exposed to low temperature. Vitamin E is a cell membrane antioxidant that protects cell integrity by reducing polyunsaturated fatty acid oxidation, whereas VC is a cytosolic antioxidant that restores the antioxidant capability of oxidized VE (Guney et al., 2007).

Previously it was found that feeding high levels of VE (200 IU / kg of feed) and Arg (1% wt / wt) improved cardiopulmonary response after an acute challenge with epinephrine compared with birds fed high levels of Arg alone in birds grown at low temperature (Ruiz-Feria, 2009). In the same work, it was found that birds fed a combination of Arg, VE, and VC had a better cardiopulmonary response than birds fed either a combination of Arg and VE or a combination of Arg and VC. However, the amplification of PHS through cold stress is different from that caused by hypobaric hypoxia; in the first case, pulmonary arterial pressure (PAP) is primarily increased by an enhanced cardiac output in response to an increase in metabolic demand (Wideman, 2001), whereas in the second case, the increase in PAP is caused primarily by pulmonary vascular constriction increasing pulmonary vascular resistance (Julian, 2007). It was recently reported that feeding supplemental Arg also improved the pulmonary vascular performance of broiler chickens grown under hypoxic conditions, and its effects were further improved by the addition of the antioxidant VE and VC (Bautista-Ortega and

Ruiz-Feria, 2010).

One of the mechanisms proposed through which oxidative stress is implicated in the pathophysiological progression of PHS is by reducing availability of the NO through its reaction with superoxide to produce peroxynitrite, reducing smooth muscle vasodilation and contributing to a high PAP (Martinez-Lemus et al., 1999; Tan et al., 2007a; Bautista-Ortega and Ruiz-Feria, 2010). There is evidence that supplemental Arg, VE and VC improve cardiovascular performance in chickens exposed to low temperature or hypobaric hypoxia. Thus, it was hypothesized that NOX and XO are important sources of superoxide in hypoxic broiler chickens and that supplemental Arg, VE and VC modulate their activities, preserving NO. However, to our knowledge, the demonstration of NOX and XO in the vicinity of the pulmonary artery endothelium of hypoxic broiler chickens is still pending. Thus, the objectives of the present investigation were: (1) to ultracytochemicaly localize NOX and XO, (2) to semi-quantitatively determine NOX and XO activity through the quantitation of H_2O_2 deposition using scanning laser reflectance confocal microscopy, and (3) to semi-quantitatively determine the degree of oxidative stress and NO depletion using nitrotyrosine as a marker, in lung tissue of broiler chickens grown under hypobaric hypoxia or normoxia, and fed supplemental Arg, and antioxidant vitamins E and C.

MATERIALS AND METHODS

Experimental Design

One day-old chicks (Cobb 500, n = 200) were wing-banded and allocated to one of three dietary treatments: a basal control diet (CTL), containing 3,200 kcal of ME / kg of feed, 23% CP, 1.55% (wt / wt) ARG and 40 IU of VE / kg of feed; a high-ARG diet (HA), basal diet plus 0.8% (wt / wt) supplemental L-arginine HCl (SAFC Supply Solutions, St. Louis MO); or a high ARG and vitamin diet (AEC), the HA diet plus 200 IU α-tochopherol / kg of feed (Producers Cooperative Association, Bryan, TX) and 500

mg of ascorbic acid / L of drinking water (Sigma-Aldrich Corp., St. Louis, MO). The diets were iso-caloric and iso-nitrogenous and formulated to meet or exceed all of the NRC (1994) requirements. Supplemented VC in drinking water was prepared daily. The chicks were brooded conventionally with temperature starting at 32° C and decreasing 2° C each week until week 3, under a constant lighting program. The chicks were housed in six wire cages (56 cm long X 33 cm wide X 28 cm high) under normoxic conditions for 7 days and then the cages were placed in two plastic cylindrical hypobaric chambers (185 cm long and 58 cm diameter) in which the chickens were exposed to a simulated altitude of 3,000 m above sea level (oxygen partial pressure of 14.5 KPa) until they were 30 days old to amplify the incidence of PHS. The desired simulated altitude was achieved by pulling a vacuum through the chamber with a Roots Universal blower (Dresser industries, Connersville, IN). The blower was reversed to pull air through the air plenum. The chicks were kept inside the hypobaric chambers continuously except for 40 min to clean and re-feed the chicks, 15 min to decompress the chambers and 15 min to reach the desired vacuum level. The dietary groups exposed to hypobaric hypoxia were identified as: CTL-H, ARG-H and AEC-H. Additionally, one-day-old chicks were raised for 10 days under normoxic conditions (CTL-N), in an adjacent facility to the hypobaric challenge unit, and they were brooded and raised following the same procedures. The normoxic birds provided lung tissue samples as technical control for cyto- and histo-chemical studies.

Lung Tissue Collection and Sample Processing at the Microcopy and Imaging Center of Texas A&M University for Electron Microscopy Examination

A surgical plane of anesthesia was induced in the chickens by intramuscular injections of allobarbital (5, 5-diallyl-barbituric acid; 25 mg/kg of body weight). The birds were fastened in dorsal recumbency on a surgical board, the heart was exposed through an opening in the abdominal cavity and the lungs were perfused in vivo as follows. An 18-gauge hypodermic needle was inserted in the left ventricle and perfusion

was initiated by injecting 10 ml heparinized saline to dissolve blood clots followed by 10 ml of perfusion buffer which contained 1% (v / v) glutaraldehyde and 4% (w / v) paraformaldehyde in 0.1 M HEPES buffer (pH 7.4). Glutaraldehyde and formaldehyde stabilizes protein structure by establishing crosslinks and so they help maintain conformational epitopes in target proteins as well as binding sites in enzymes. The lungs were subsequently removed and placed in 10 volumes of perfusion buffer in an ice bath until further processing. The lungs were subsequently fixed for 1 h at 4 °C in cold 5.0 % (v / v) acrolein in 0.1 M HEPES buffer (pH 7.4). Acrolein is an excellent for cytochemistry and immunlabeling fixation and is an alternative to glutaraldehyde and formaldehyde. Specimens were washed overnight at 4 °C in cold 0.1 M HEPES buffer (pH 7.4) plus 5% (weight/vol) sucrose and 1% (vol/vol) dimethylsulfoxide. Washes are the most critical factor after fixation and the washing buffer contains protective components (e.g. sucrose). Biological tissues fixed using the aforementioned procedure can be safely further processed for light or electron microscopy studies.

Cytochemical Localization of XO and NOX

The cytochemical localization of NOX and XO was conducted as described by Ellis and Grant (2002). For the localization of XO, the lung samples that were previously processed for electron microscopy were brought to room temperature in the final two buffer washes which contained 0.1 M glycine. The samples were then preincubated 2 times for 6 min at 37°C (temperature range 37°C to 42°C) using a microwave set to 200 watts and in the following medium: 10 mM cerium chloride, 10 mM 3-amino-1, 2, 4-triazole, 0.1 M HEPES buffer (pH 8), 7% sucrose, and 0.0002% Triton X-100. They were then incubated in the following complete reaction medium: 10 mM cerium chloride, 10 mM 3-amino-1, 2, 4-triazole, 10 mM hypoxanthine, 0.1 M HEPES buffer (pH 8), 7% sucrose, and 0.0002% Triton X-100 (see in Figure 4A a depiction of a complete reaction conducted for NAD(P)H-oxidase at a tissue level). The reaction during the incubation was performed 4 times for 6 min using a microwave as described

above. The reaction was terminated by washing the sample twice in cold 0.1 M HEPES buffer (pH 7.4) plus 5% sucrose. In the case of NOX localization, the following preincubation medium was used: 2.0 mM cerium chloride, 10 mM 3-amino-1, 2, 4-triazole, 0.1 M HEPES buffer (pH 7.4), 7% sucrose, and 0.0002% Triton X-100. The samples were then incubated in the following complete reaction medium: 2.0 mM cerium chloride, 10 mM 3-amino-1, 2, 4-triazole, 0.8 mM NADH, 0.1 M HEPES buffer (pH 7.4), 7% sucrose, and 0.0002% Triton X-100 (Figure 4A).

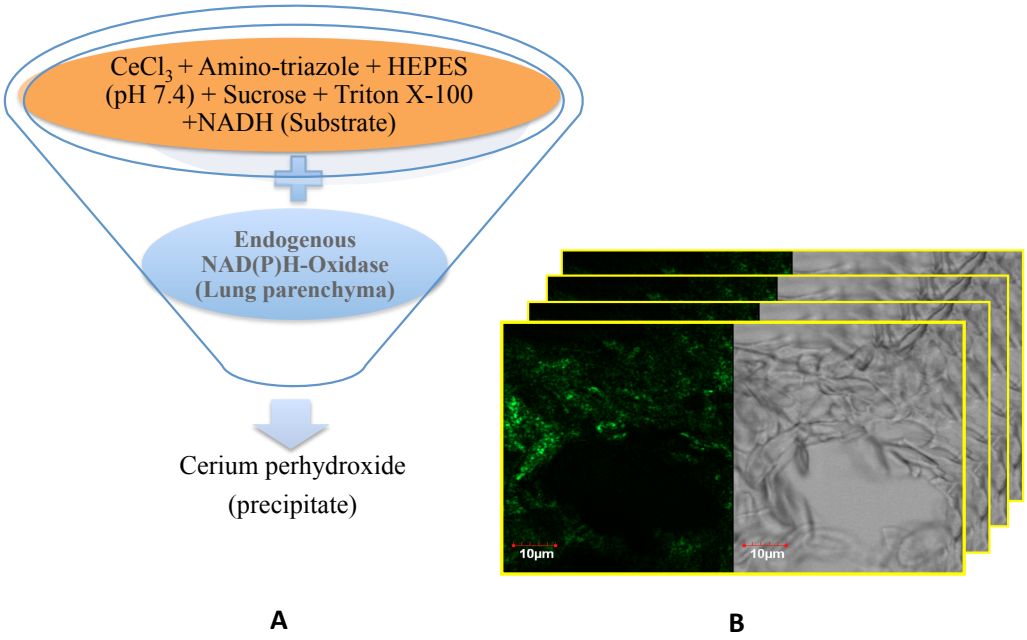


Figure 4. Schematics depicting the cerium-based method used to localize oxidases and to assess their enzymatic activity. A. Reagents added to the complete reaction buffer used to localize NAD(P)H-oxidase and assess it activity. B. Cerium perhydroxide deposits (green areas) on a cryostat lung section reflecting light from a laser beam. Olympus FV100 confocal microscope, 10 X objective, reflectance mode by 488 nm laser.

Specificity of the reactions were demonstrated by: (1) omitting the substrate (hypoxanthine or NADH); and (2) using an inhibitor of XO (1.0 mM allopurinol) or an inhibitor of NOX (1.0 mM diphenyleneiodonium chloride, DPI). Specimens were then post-fixed overnight in 1% (weight/volume) osmium tetroxide, dehydrated, infiltrated

and embedded. Grids were examined and photographed at 100 kV in the transmission electron microscope (JOEL JSM-6400). With this method, the intact active site of the oxidases (XO and NOX) is preserved and when it is reacted with the substrate the enzyme produces superoxide. In turn, superoxide is dismutased by superoxide dismutase to hydrogen peroxide. Hydrogen peroxide then reacts with cerium chloride, in the presence of aminotriazole that inhibits endogenous catalase and peroxidases, to produce a water-insoluble (electron dense) precipitate cerium perhydroxide (Figure 4B).

Cryostat Sectioning of Lung Samples

Five mm-thick cross sections were cut from each lung and then equilibrated in 30% sucrose in 0.1 mM HEPES (pH 7.4) overnight at 4 °C. Frozen lung tissue samples were then cut to a thickness of 20 μm and mounted as suggested by Robinson and Batten (1990). In short, the coverslips were rinsed twice in the fixation buffer and then mounted on glass slides in glycerol/0.1 M HEPES buffer (50 %/50%), pH 7.4, and then mounted on microscope slides for immediate observation.

Histochemical Localization of XO and NOX

Histochemical localization of XO and NOX in the lung parenchyma was done as described above for the cytochemical localization of both enzymes with the difference that the pre-incubation and incubation media were scaled up to accommodate several mounted specimens to be reacted successfully. In this case, the amount of cerium perhydroxide that precipitates in the lung parenchyma correlates with the activity of XO or NOX. The oxidase activity is then measured by the average reflectance intensity of a laser beam casted on the mounted tissue sample using a confocal microscope. For XO, the technical control consisted in 2 mM allopurinol added to the pre-incubation and incubation media. Treatment with 2 mM allopurinol inhibited 82 % of the XO activity. In the same samples, incubation with higher allupurinol concentrations (10 mM

allopurinol) did not increase inhibition XO activity. We decided to conduct *in situ* determination of oxidase activity in fixed intact lung parenchyma because these procedures preserve active sites of enzymes while maintaining tissue / cell structure so as to provide a picture as close as possible to what happens in the live bird. Also, in the present investigation we used the cerium-based method to assess oxidase activity in intact lung tissue of broiler chickens; this method has been well established to demonstrate oxidase activity in cells (Robinson and Batten, 1990; Ellis and Grant, 2002). In the cerium-based method, oxidases present in the intact lung parenchyma sample (i.e. enzymes preserve its active sites when tissues are properly fixed) were reacted with its substrate (i.e. hypoxanthine for XO and NADH for NOX) to produce superoxide, which in turn was dismutased by tissue superoxide dismutase to produce hydrogen peroxide. The addition of aminotriazole to the reaction medium inactivated catalase and peroxidases present in the tissue thus sparing the hydrogen peroxide that had been produced; hydrogen peroxide was then reacted with cerium chloride to produce cerium perhydroxide. The specificity of this staining (i.e. cerium perhydroxide precipitates) was demonstrated by the fact that perhydroxide deposits were only seen in cell populations whereas no staining was detected in the lumen of small vessels (Figure 4B).

Determination of NOX and XO Activity

The specimens were examined with an Olympus FV1000 confocal microscope. The reflective mode was used with the laser (λ = 488 nm). Routine observations were made by simultaneously generating a phase-contrast image on a split screen. The digitalized images were captured by computer and analyzed using the software (Image J). Since the majority of reflectance units were deemed to have come from the XO activity, the direct readings of reflectance units were presented.

Immunocytochemical Demonstration of Nitric Oxide Depletion in Lung Tissue

Sections from lungs in which XO had been localized were picked up on nickel grids, oxidized for 6 min with 2% (weight/volume) periodic acid in a microwave cycle MW1 (2 min ON, 2 min OFF, 2 min ON) followed by four 1 min washes in phosphate buffered saline (PBS, pH 7.2) in a microwawe cycle MW2 (1 min ON). Grids were floated on PBS blocker [PBS plus 4% (weight/vol) cold water fish gelatin, (Sigma Chemical Co., St. Louis, MO)] and the microwave cycle MW1 was run. The grids were then floated with a rabbit polyclonal anti-nitrotyrosine antibody (Millipore Corporation, Billerica, MA) diluted 1:50 with PBS blocker and reacted by running a MW1 cycle. After 2 washes with PBS blocker, grids were washed twice with Tris–HCI saline buffer (pH 7.6) (TBS) plus 4% (weight/vol) plus cold water fish gelatin, again each wash was done running a MW2 cycle. Grids were floated with donkey anti-rabbit IgG labeled with 12 nm colloidal gold (Jackson Immunoresearch Labs Inc., West Grove, PA) diluted 1:30 with TBS blocker and reacted with a MW1 cycle. Grids were washed 3 times with TBS blocker, followed by 3 washes with deionized water on MW2 cycles. Control grids were incubated with only the gold-labeled secondary antibody. Data on NO depletion was presented as the number of immunogold particles / 100 μm^2.

Determination of XO and NOX Activity in Lung Homogenates

Frozen lung samples (0.5 g) were homogenized in 0.5 ml of ice-cold 50mM Tris-HCl buffer (pH 7.4) containing 10 µg/ml antipain, 1mM EDTA and 1mM DTT to avoid oxidative conversion of xanthine dehydrogenase to xanthine oxidase, centrifuged at 10,000 X *g* for 10 min at 4°C and then frozen at -80°C until assayed. Homogenate protein content was determined using an assay kit based on the Bradford method, which takes advantage of the color change of Coomassie® dye when it binds to proteins in acidic medium. When the dye binds, there is an immediate shift of the absorption

maximum from 465 to 595 nm with a simultaneous change in color from brown to blue. The assay was conducted according to the manufactures instructions. (Cayman Chemical Company, Ann Arbor, MI).

NAD(P)H oxidase activity in lung tissue homogenates was assessed through the oxidation of added NADH oxidase-generated superoxide (Vincent et al., 2007). On a white 96-well plate, a 50 µl sample was mixed with 80 µl Tris-HCl with either 20 µl additional Tris-HCl or 20 µl 1 mM diphenyliodonium (DPI; final concentration 100 µM) to inhibit NOX oxidase. The reaction was started by adding 50 µl of 600 µM NADH solution to a final concentration of 150 µM NADH and kinetics of the reaction curve were sampled every 1 min for 10 min. The decrease in absorbance in the presence of DPI was subtracted from the decrease in absorbance in the absence of DPI to give a measure of oxidation of NADH by NOX (NOX activity measured as a change in absorbance units per minute). Subsequently, the volumetric NOX activity was calculated (U / L) as follows, using an extinction coefficient (ε) of 6.22 L / mM / cm (Fasman, 1989) as shown next:

Volumetric NOX activity (U / L) = Absorbance at 340 nm * 1000 * dilution * volume / ε * homogenate volume. The specific NOX activity was reported as U / mg protein (Fasman, 1989).

Xanthine oxidase activity was determined in lung homogenates using an assay kit based on a multistep enzymatic reaction where the end product was resorufin, a highly fluorescent compound, which was subsequently analyzed using an excitation wavelength of 520 nm and an emission wavelength of 595 nm. The XO activity measure by the kit was in ìU / ml homogenate (Cayman Chemical Company), from which specific XO activity was calculated based on protein determination in the lung homogenates and presented as ìU / mg protein.

Determination of Thiobarbituric Acid Reactive Substances **(TBARS)**

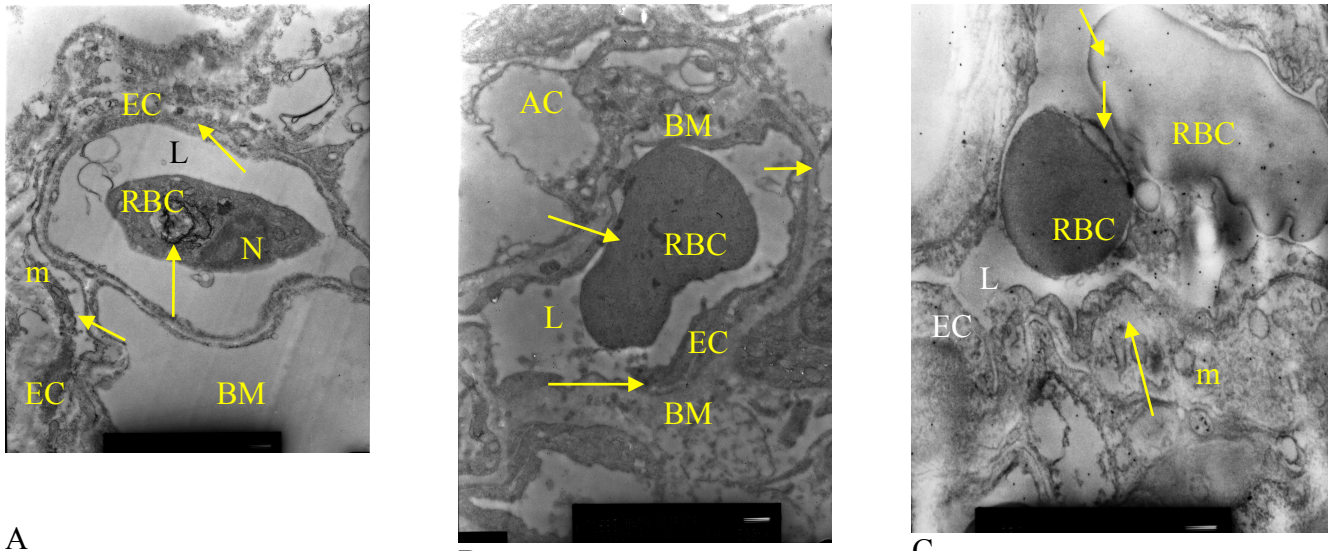
Vacutainers containing EDTA were used to collect 2 mL of blood from 8 healthy

birds in each group. The blood was centrifuged at 1,500 X *g* for 5 min; plasma was collected in labeled tubes and stored at −80 °C until analysis. Lipid peroxidation was determined by measuring the level of malonyl dialdehyde (MDA) using the Ohkawa technique (Ohkawa et al., 1979) following the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI). This method is based on the reaction of thiobarbituric acid with MDA. MDA dilutions ranging between 0 and 50 μM were used as standards. Results were measured at 532 nm using a plate reader (Biotek Instruments, Inc., Vermont, USA).

RESULTS AND DISCUSSION

Cytochemical Localization of Xanthine- (XO) and NAD(P)H-Oxidase (NOX)

Both enzymes, NOX (Figure 4A and 1B) and XO (4C), were localized close to the pulmonary vascular endothelial cell of CTL-N and hypoxic chickens. In the CTL-N chickens, NOX localized within vesicles, outer mitochondrial membrane as well as in the cell membrane of endothelial cells (Figure 5A). Hypoxic chickens from all dietary treatments also showed NOX localized in endothelial cell junctions (Figure 5B). XO localized in similar cellular domains as NOX, as well as in the cell membrane of red blood cells (Figure 5C).



A B C

Figure 5. NAD(P)H-oxidase (NOX) and Xanthine oxidase (XO) in broiler chicken lung histological sections. For NOX, sections were reacted with 2.0 mM cerium chloride, 10 mM 3-amino-1, 2, 4-triazole and 0.8 mM NADH. For XO, sections were reacted with 10.0 mM cerium chloride, 10 mM 3-amino-1, 2, 4-triazole and 1 mM NADH. A. 7 d old normoxic chicken. H_2O_2 deposition (arrows) is evident in outer membrane of mitochondria of RBC and EC, and vesicles in the EC. B. 28 d old hypoxic chicken. H_2O_2 deposition (arrows) is evident in EC junctions. C. 28 d old hypoxic chicken. H_2O_2 deposition is evident (arrows) in EC membrane (facing the lumen and BM) and RBC membrane. AC = air capillary; EC = endothelial cell; L= lumen; RBC = red blood cell; BM = Basement membrane, N = Nucleus; m=mitochondria. The bar indicates 500 nm (Transmission Electron Microscope).

Although XO and NOX have been associated with vascular diseases, including PH in humans (Warwick et al., 2008; Guzik and Griendling, 2009) and in the rodent model (Zalba et al., 2000; Jankov et al., 2008), in chickens the role of these enzymes has been less studied, especially in the pulmonary artery endothelial, the tissue where the primary lesion in chicken PHS occurs. To our knowledge, this is the first time that NOX and XO are demonstrated ultrastructurally in the intact lung of hypoxic and normoxic broiler chickens. These results suggest that both enzymes may be important sources of ROS in the vicinity of chicken pulmonary artery endothelial cell.

The results on NOX and XO localization agree with previous reports that showed NOX activity confined to cell membrane rafts or within endosomal / phagosomal compartments (Terada, 2006; van der Vliet, 2008), whereas previous reports indicated that XO is attached to the membrane of red blood cells (Al-Khalidi and Chaglassian, 1965); these authors detected negligible XO activity in the serum and white blood cells; so the XO activity that they determined in the whole blood was attributed to that present in red blood cells. In addition, our findings that XO was localized within vesicles on endothelial cells agrees with the results by Poss et al., (1996), who reported that intracellular XO was contained in organelles, possibly endosomes.

In Situ NOX and XO Activities in Intact Lung Parenchyma

The results on the effect of supplemented Arg, VE and VC on *in situ* NOX and XO activity, assessed through the average reflected light intensity of cerium perhydroxide precipitates, in intact lung parenchyma of broilers exposed to chronic hypobaric hypoxia as well as in the CTL-N chickens are presented in Figure 6. Chickens in the CTL-H group had lower ($P < 0.05$) XO activity than birds in the AEC-H and CTL-N groups, but not different from chickens in the ARG-H group. Although chickens in the AEC-H group had a higher ($P < 0.05$) XO activity than birds in the CTL-H group, the AEC-H birds still had a lower XO activity compared with birds in the CTL-

N group. In general, hypoxic chickens, regardless of dietary treatment, showed a lower ($P < 0.05$) XO activity than the CTL-N birds. To our knowledge, no work has been published using an *in situ* approach to study the effects of diet on oxidase activity, namely XO and NOX, in relation to PHS in broiler chickens.

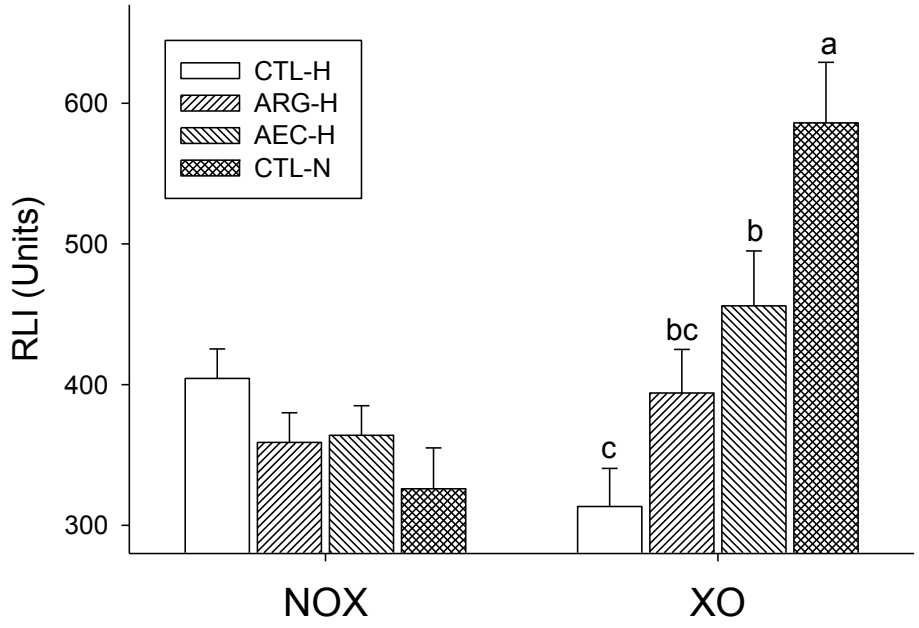


Figure 6. Xanthine- (XO) and NADH-oxidase (NOX) activities measured through the average reflected light intensity (RLI, confocal microscopy) of perhydroxide deposits in lung tissue of hypoxic (H, 28 d old, n = 5-6 per dietary treatment) or normoxic (N, 10 d old, n = 3) broiler chickens. Oxidases in cryostat sections were reacted with 2.0-10.0 mM cerium chloride, 10 mM 3-amino-1, 2, 4-triazole and 0.8 mM NADH (NOX) or 10mM hypoxanthine (XO). Hypoxic birds were fed a control diet (CTL, commercial corn-soybean meal based diet), a high arginine diet (HA, control diet plus 0.8% arginine); or a high arginine and vitamin diet (AEC, HA plus vitamin E at 200 IU / Kg of feed and vitamin C at 500 mg / L of drinking water); normoxic birds were fed the control diet. [abc] Means with a different letter for the same enzyme differ significantly ($P < 0.05$).

Previously, we showed that supplemental Arg and antioxidant vitamins E and C improved cardiovascular performance in broiler chickens exposed to chronic hypobaric hypoxia or cold temperatures (Ruiz-Feria, 2009; Bautista-Ortega and Ruiz-Feria, 2010).

In these investigations, we suggested that antioxidant vitamins reduced the effects of ROS produced by XO and NOX, whereas supplemental Arg ensured enough substrate for NO production, resulting in a healthier endothelium and better vasodilation.

However, we did not have information regarding the distribution of XO and NOX nor on their activity in hypoxic chicken lung cell populations, including the pulmonary artery endothelium. In the present study, the XO activity in the lung parenchyma (composed of different anatomic elements including small vessels, small arteries and parabronchial walls) may be related to local stimuli (i.e. hypoxia) in parenchymal cell populations as well as from circulating XO attached to the cell membrane of red blood cells and endothelial cell. For example, Poss et al., (1996) demonstrated that mammalian vascular endothelium, fibroblast and type 2 epithelial cells incubated with 5 mU / ml XO, showed increased cell-specific XO activity, indicating that those cells were able to take up XO from the medium. In addition, it has been demonstrated that XO released from XO-rich areas such as the splanchnic tissue attaches to the endothelium surface through sulfate proteoglycans (Houston et al., 1999).

In the present work, exposure to hypobaric hypoxia led to a reduction in XO activity, which is in contrast with the response of mammalian endothelial cell cultures exposed to either severe or moderate hypoxia (Poss et al., 1996; Kelley et al., 2006). Also, chickens respond differently from humans to dietary allopurinol, an inhibitor of XO; while in allopurinol-treated humans a reduction in XO activity is related to improved PH symptoms (Pacher et al., 2006), allopurinol-fed normoxic chickens showed signs of toxicity manifested as significant body weight loss and increased oxidative stress (Carro et al., 2010). In the present investigation hypoxic chickens had a 60% lower average light intensity (235.7 ± 32 reflectance units) than the normoxic controls (586 ± 43 reflectance units). A reduction in XO activity may indicate a decrease in uric acid level and so a concomitant decrease in antioxidant capacity; for instance it has been reported that a 33% decrease in uric acid production in birds leads to an increase in oxidative stress (Stinefelt et al., 2005).

The results presented here also show that Arg and antioxidant vitamin supplementation had a tendency to restore XO activity to levels found in young chickens grown under normoxia, suggesting that the nutritional supplementation slowed the negative effects of hypobaric hypoxia on XO activity, which may be associated with an increase in uric acid. Uric acid may function as an antioxidant in the plasma, however it may switch to pro-oxidant (urate radical) when other antioxidants become limited. It has been shown that the prooxidant capability of UA is reversed by increasing levels of VC (Abuja, 1999). In addition, VC has been shown to restore the antioxidant capability of VE (Guney et al., 2007) and uric acid. Thus, uric acid may have ameliorated the potential harmful effects of an increased superoxide production observed in supplemented groups of birds in the present study. Furthermore, the results on *in situ* XO activity were similar to those obtained on XO activity in lung homogenates (Table 2); there was significantly higher XO activity in the CTL-N group of chickens compared with birds in the hypoxic groups (Table 2). Among the birds raised under hypoxic conditions, the XO activity was higher in the AEC-H than in the CTL-H group, whereas XO had intermediate levels in ARG-H birds.

Table 2. Effect of diet and hypobaric hypoxia on parameters related to pulmonary hypertension (right ventricle to total ventricle weight ratio, RVW/TVW; hematocrit, Hc, %), and NADH (NOX, U / L) or xanthine oxidase (XO, μU / g of protein) activity in lung homogenates of broiler chickens.

Parameter	Treatment			
	CTL[1]- H[2]	ARG-H	AEC-H	CTL-N
RVW/TVW ratio	0.26±0.031[a]	0.26±0.006[a]	0.27±0.009[a]	0.18±0.013[b]
% Hc	39±4.6[a]	41±1.9[a]	39±3.3[a]	27±0.9[b]
NOX	1.59±0.42	1.36±0.42	2.0±0.42	1.49±0.42
XO	0.43±0.6[b]	0.66±0.6[b]	1.21±0.6[b]	3.87±0.6[a]

[1]CTL, basal diet containing 3,200 kcal of ME / kg of feed, 23% CP, 1.55% (wt / wt) ARG and 40 IU of VE / kg of feed; HA, a high-ARG diet, CTL diet plus 0.8% (wt / wt) supplemental L-arginine HCl; AEC, high ARG and antioxidant vitamin diet (AEC), the HA diet plus 200 IU α-tochopherol / kg of feed and 500 mg of ascorbic acid / L of drinking water.

[2] H, hypoxic birds raised at simulated 10,000 ft (3,000 m above sea level); N, normoxic birds raised at sea level in a pen next to the hypobaric chamber.

[a,b] Means within a row, and within the same parameter, lacking a common superscript differ ($P \leq 0.05$)

NOX activity was not affected by dietary or hypoxia conditions in clinically healthy birds (Figure 6). It has been reported that in endothelial cell in vitro NOX activation is necessary for increased XO activity and superoxide production in a NOX-dependent manner (McNally et al., 2003). Our results do not agree with this observation because we did not find any differences in NOX activity among dietary treatments whereas they differ in XO activity. In this regard, it is possible that in our in vivo study the exposure of the birds to hypobaric hypoxia activated NOX in the lung parenchyma immediately, which in turn induced the activity of XO that we detected at 28 d. The NOX activity measured in the lung homogenates showed no effect caused by dietary treatment or hypoxia (Table 2), agreeing with the results obtained using the *in situ* approach.

One of the limitations of the present study was that the assessment of the activity of the different NOX's isoenzymes expressed in parenchymal cell populations was not performed. However it has been reported that analysis of total lung or airway mRNA revealed the presence of a substantial amount of NOX isoform 2 (NOX2), dual oxidase (DUOX) 1, and DUOX2, and low amounts of NOX1 and NOX4. In addition, in the lung, expression of NOX primarily originates from lung macrophages (NOX 2), however other NOX/DUOX isoenzymes are largely expressed in non-phagocytic cells within the lung such as airway epithelium, pulmonary endothelial cells, fibroblasts and smooth muscle cells (Lassegue and Griendling, 2010).

Effect of Supplemented Arg, VE And VC on Nitric Oxide Depletion (Nitrotyrosine Immunoreactivity)

There was no difference in nitrotyrosine levels among birds grown under hypobaric hypoxia, regardless of dietary treatment, or compared with birds grown under normoxia (Figure 7). Nevertheless, we found that birds with clinical signs of PHS (i.e. ascitic fluid in the abdominal cavity and high RVV:TVW ratio) showed higher ($P < 0.001$) levels of peroxynitrite formation in the vascular endothelium and its vicinity

(Figure 8), indicating a higher oxidative stress chickens with PHS than in clinically healthy hypoxic chickens.

In the present study we focused on clinically healthy broiler chickens, however some birds from the different experiment groups developed PHS. Data from all birds developing PHS were combined and then compared with data from clinically healthy broilers. We deemed such comparison important to test the effectiveness of the technique for detecting oxidative stress associated with PHS birds. In this regard, we confirmed previous reports that PHS broiler chickens have elevated oxidative stress (Nain et al., 2008a,b).

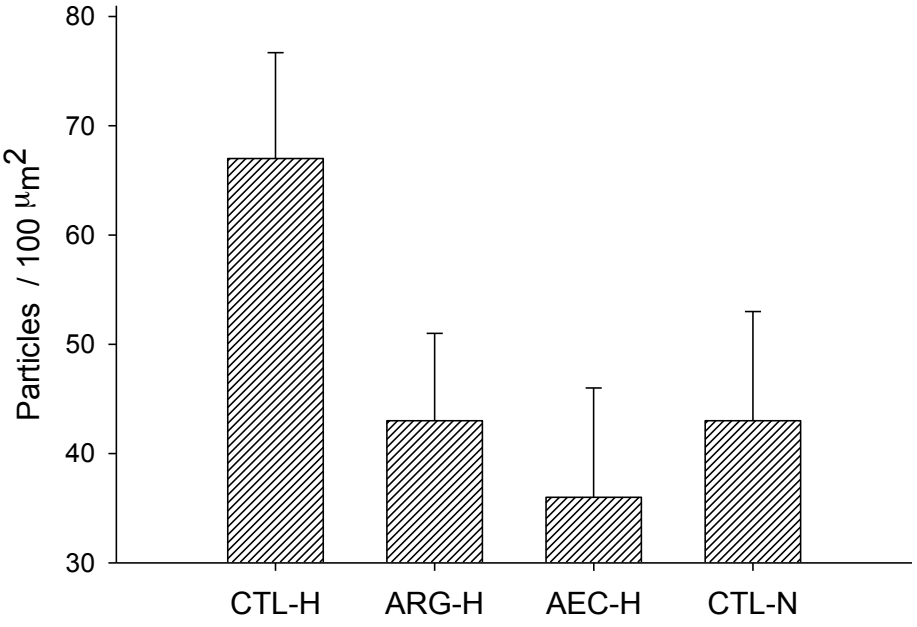


Figure 7. Nitrotyrosine, an indicator of oxidative stress and nitric oxide depletion, in the lung parenchyma of clinically healthy broiler chickens grown under hypoxia (H) or normoxia (N). Lung tissue samples, processed for electron microscopy, were picked up in nickel grids and were reacted with the following reagents in the order shown: 2% periodic acid, polyclonal rabbit anti-nitrotyrosine antibody, blocked with 4% fish gelatin, donkey anti-rabbit antibody labeled with 12 nm colloidal gold. Control grids were incubated with only gold-labeled secondary antibody.
Hypoxic birds were fed a control diet (CTL, commercial corn-soybean meal based diet), a high arginine diet (ARG, control diet plus 0.8% arginine); or a high arginine and vitamin diet (AEC, ARG plus vitamin E at 200 IU / Kg of feed and vitamin C at 500 mg / L of drinking water); normoxic birds were fed the control diet. n= 3, 4, 2 and 3 for the CTL, ARG and AEC groups, respectively.

Once formed, NO can react with a variety of species in the vasculature, including hemoglobin (forming iron-nitrosyl-hemoglobin or nitrate), superoxide (forming peroxynitrite), ROS, reactive nitrogen species, soluble guanilyl cyclase, and cytochrome C oxidase (Chen et al., 2008). Although NO can react with several molecules, we were interested in its reaction with superoxide because protein tyrosine nitration is an established biomarker of oxidative stress and the molecular signal of peroxynitrite formation during development, oxidative stress and aging (Yeo et al., 2008).

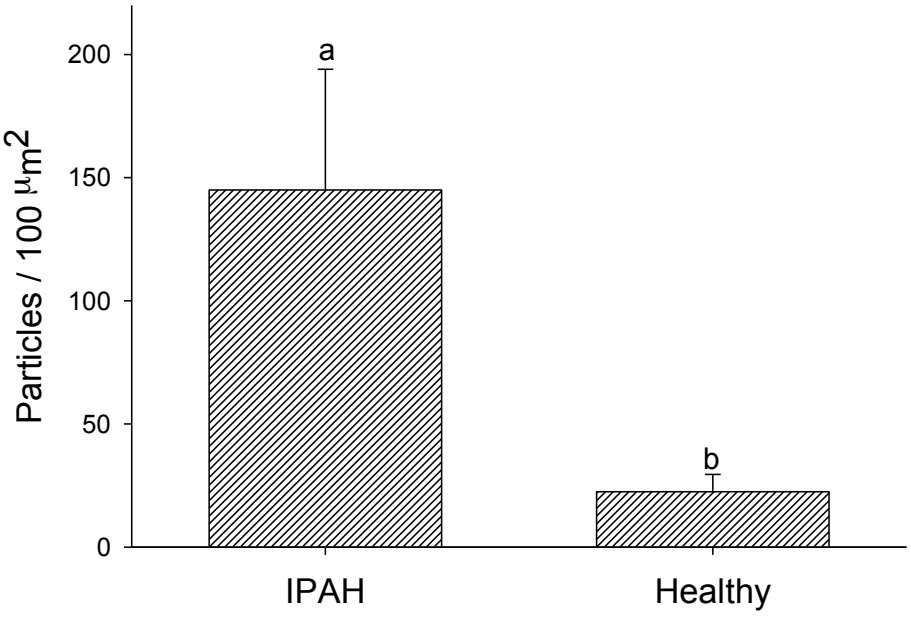


Figure 8. Nitrotyrosine concentration, an indicator of oxidative stress and nitric oxide depletion, in the lung parenchyma of clinically healthy chickens (Healthy; n = 9) and chickens with clinical signs of pulmonary hypertension (IPAH; n = 3). Lung tissue samples, processed for electron microscopy, were picked up on nickel grids and were reacted with the following reagents in the order shown: 2% periodic acid, polyclonal rabbit anti-nitrotyrosine antibody, blocked with 4% fish gelatin, donkey anti-rabbit antibody labeled with 12 nm colloidal gold. Control grids were incubated with only gold-labeled secondary antibody.
[a,b] Means with different letter differ significantly ($p<0.05$).

With this in mind, in the present study the reaction between NO and superoxide that took place in the live bird led to the formation of peroxynitrite; in turn peroxynitrite

reacted with tyrosine residues of proteins (formation of nitrotyrosine), which were then labeled with immunogold particles.

The reaction between superoxide and NO disturbs the steady state concentration of NO; superoxide generated either intracellularly or extracellularly during NO generation results in a concomitant increase in oxidative intermediates with a decrease in steady-state NO concentration and a proportional reduction in the levels of soluble guanilyl cyclase, ERK, HIF-1a, and p53 regulation (Thomas et al., 2006). Our results show that in chickens that developed PHS there was a higher number of NO molecules reacting with superoxide molecules and were subsequently lost to peroxynitrite at the expense of NO bioavailability. Peroxynitrites are reactive species of nitrogen and oxygen that modify biomolecules, including DNA, lipids, and proteins. In addition, peroxynitrite reacts with several chemicals, including metal centers and CO_2. The end result of this reaction is cell and tissue damage (Yeo et al., 2008).

Effect of Supplemented Arg, VE and VC on PHS-Related Parameters

Hematocrit (%) is used as an indicator of hypoxemia (low oxygen tension in the blood) because when chickens are exposed to environmental chronic hypoxia there is an increase in the production of red blood cells. As expected, hypoxic broilers, regardless of diet, had higher hematocrit than chickens grown under normoxia (data not presented). Although PHS broilers presented almost 4 percentage units higher hematocrit than the healthy looking hypoxic birds, this difference did not reach statistical significance probably reflecting a high biological variation (Table 3).

The RVW/TVW ratio is commonly used as a measure of the hypertension level, which directly causes right ventricular hypertrophy in broiler chickens. This ratio provides a convenient standard whereby chickens of different age and size can be compared (Wideman, 2001). Clinically healthy domestic fowl with normal pulmonary arterial pressure have RVW/TVW ratios ranging from 0.15 to 0.27, whereas sustained pulmonary hypertension causes RVW/TVW ratios above 0.28 (Wideman, 2001). There

were no differences in RVW/TVW ratio among hypoxic broiler chickens from the different dietary treatments. However, hypoxic broilers were significantly more hypertensive ($P < 0.05$) that control normoxic broiler chickens (Table 3). In addition, broilers that developed PHS were significantly ($P < 0.05$) more hypertensive than hypoxic healthy looking broiler chickens (Table 3).

In general, the results on hematocrit and RVW/TVW ratio showed a similar trend in the groups studied because the two parameters are physiologically related: a high hematocrit will lead to a high RVW/TVW ratio. These results agree with those reported elsewhere (Bautista-Ortega and Ruiz-Feria, 2010) using a larger population of broilers exposed to hypobaric hypoxia.

Table 3. Pulmonary hypertension (PHS)-related parameters in PHS and healthy looking hypoxic broiler chickens.

PHS parameter	Group	
	PHS[1]	Hypoxic Healthy looking
RVW/TVW ratio	0.42±0.060[a]	0.26±0.090[b]
Hematocrit (%)	43±5.0	40±1.6

[1]PHS, birds that had ascitic fluid accumulated in the abdominal cavity at the time of sampling; RVW/TVW = right ventricle weight/total ventricular weight ratio

[a,b] Means within a row, and within the same parameter, lacking a common superscript differ ($P \leq 0.05$)

The fact that there were no differences in the parameters mentioned above might be because of the exposure to a simulated of 3,000 m (10,000 ft) above sea level, which was very severe. Such chronic exposure elicited similar erythropoiesis activity and subsequent lead to similar degrees of hypertension in birds from the different dietary treatments.

Effect of Supplemented Arg, VE and VC on Lipid Peroxidation (TBARS) in the Plasma

There was no effect of dietary treatment on plasma TBARS level in plasma samples from hypoxic broiler (data not presented). Levels of peroxidation in the plasma represent the physiological status of the whole bird tissues. Our results suggest that the broiler chickens from the different dietary treatments that were subjected to hypobaric hypoxia suffered from the same level of oxidative stress in agreement with the results on peroxynitrite presented earlier.

In summary, XO and NOX localized in similar domains in the pulmonary artery endothelium. Moreover, supplemental Arg and antioxidant vitamins E and C did not have an effect on NOX activity nor on oxidative stress in the vicinity of pulmonary artery endothelium. However, supplemented Arg, VE and VC restored XO activity that had been suppressed by exposure to hypobaric hypoxia. The mechanism (s) through which antioxidant vitamins modulate XO activity in the broiler chicken remains to be determined. The dual role of XO, which produces superoxide and uric acid (antioxidant), may have buffered the effects of superoxide produced concomitantly in clinically healthy birds from the supplemented group. In this group, the antioxidant capability of uric acid and VE may have been restored by VC.

CHAPTER V

L-ARGININE AND ANTIOXIDANT VITAMINS, E AND C, REDUCE PULMONARY ARTERIAL REACTIVITY TO PHENYLEPHRINE

INTRODUCTION

Pulmonary hypertension syndrome (**PHS**; also known as idiopathic pulmonary arterial hypertension: **IPAH**) is a metabolic disorder that affects fast-growing broiler chickens. It is generally accepted that in susceptible broiler chickens, this disorder is initiated by an increase in either cardiac output (e.g., because of increased metabolic rate) or vascular resistance to blood flow (e.g., exposure to hypobaric hypoxia) (Julian, 2007). Such triggering factors of PHS lead to a series of pathophysiological changes including endothelial dysfunction and vascular remodeling that culminates with the death of affected birds, typically from right ventricular failure caused by sustained pulmonary hypertension.

Chronic hypoxia alters reactivity of pulmonary arteries (**PA**) from mammals and birds. For example, chronic hypoxia led to vascular remodeling and an increased receptor-dependent vasoconstriction (thromboxane mimetic U46619) in intrapulmonary arteries (100-140 μm diameter) from pigs (Kelly et al., 2005). In addition, hypoxia has been shown to either have no effect (Kelly et al., 2005) or decreased receptor-dependent vasodilation in intrapulmonary arteries (Tulloh et al., 1997; Alvarez-Medina, et al., 2011). In pulmonary arteries from hypertensive broiler chickens exposed to hypobaric hypoxia, acetylcholine induced a higher vasodilation than in non-hypertensive age mate broilers with no effects on contraction induced by phenylephrine (**PE**; Alvarez-Medina et al., 2011). The apparent contradictions regarding the effect of hypoxia on vasocontrictors in pulmonary artery seems to vary depending on the size of the artery, the species and experimental set up (e.g. ex vivo versus in vitro).

The primary mechanisms leading to endothelial dysfunction and vascular remodeling in IPAH remain to be identified (Stenmark et al., 2009). In healthy individuals, a balance between vasoconstrictors (e.g. endothelin-1) and vasodilators (e.g. nitric oxide; **NO**) (Luscher et al., 1990) maintains basal tone. However, in IPAH this balance is upset (endothelial dysfunction) so that the contribution of vasodilators is reduced and/or that of vasoconstrictors is increased, with vasoconstrictors usually exerting a dominant effect.

The onset of PHS has been associated with an increased production of reactive oxygen species (**ROS**) and endothelial cell damage (Enkvetchakul et al., 1993; Wedgwood and Black, 2003; Pan et al., 2007; Nain et al., 2008a,b). Consistent with the hypothesis that ROS play a role in PHS, there is evidence of the synergistic effect of supplemental L-arginine (**Arg**) and vitamins E (**VE**) and C (**VC**) on the cardiovascular performance of hypertensive broiler chickens (Lorenzoni and Ruiz-Feria, 2006; Ruiz-Feria, 2009; Bautista-Ortega and Ruiz-Feria, 2010). Arginine is the substrate for eNOS, enzyme that produces NO and so potentiates vasodilation. Vitamin E is a cell membrane antioxidant that protects cell integrity by reducing polyunsaturated fatty acid oxidation, whereas VC is a cytosolic antioxidant that restores the antioxidant capability of oxidized VE (Guney et al., 2007). These antioxidant vitamins may neutralize ROS, which readily react with NO to produce peroxynitrite, thus sparing NO and potentiating vasodilation. Uric acid (**UA**) is produced by the enzyme xanthine oxidoreductase from xanthine and hypoxanthine, which in turn is produced by the breakdown of purines. It has been reported that there is a negative relationship between plasma/serum UA concentrations and oxidative stress in the chicken (Carro et al., 2010), suggesting that UA may act as an antioxidant in the chicken.

Previously, we reported that chronic supplementation with Arg, VE and V**C** improves pulmonary vasodilation after a bolus injection with epinephrine compared with birds fed regular diets (Lorenzoni and Ruiz-Feria, 2006; Ruiz-Feria, 2009; Bautista-Ortega and Ruiz-Feria, 2010). Catecholamines play an integral role in the control of peripheral vascular resistance (i.e. as a result of vasoconstriction) and blood flow

distribution through the activation of α- and β-adrenoceptors (Guimarães and Moura, 2001). Upon challenge, endothelium-dependent vasodilation (e.g. via NO) is activated by catecholamines directly or secondary to vasoconstriction to restore vascular tone (Guimarães and Moura, 2001). Under in vivo conditions there is the possibility of indirect contributions from neuronal, endocrine, paracrine sources or even metabolism in adjacent tissues (Russell et al., 2008), thus, one of the questions that arose from our previous work was whether the direct synergistic effect of Arg, VE and VC on vascular performance would be maintained in the isolated pulmonary artery of hypoxemic broiler chickens.

In the present investigation, the effects of Arg and antioxidant VE and VC on vascular reactivity to PE (an α1-adrenergic receptor agonist) were examined in isolated pulmonary arteries from hypoxemic male broiler chickens. It was hypothesized that the hypoxemic/hypertensive pulmonary arteries have altered vascular reactivity (increased) to PE where a reduced NO may play an important role. We further hypothesized that supplemental Arg potentiates NO production whereas VE and VC protect NO, thus restoring PA reactivity (**PAR**) in the hypoxemic chickens to levels similar to those in normoxic ones.

MATERIALS AND METHODS

Experimental Design

One-day-old Cobb 500 broiler chicks were used to study the effect of dietary Arg, VE, and VC on pulmonary artery reactivity to PE and parameters related to PHS. The chicks were wing-banded and were randomly allocated to one of three dietary treatments: a control diet (**CTL**), containing 3,200 kcal of ME / kg of feed, 23% CP, 1.55% (wt / wt) Arg and 40 IU of VE (α-tochopherol) / kg of feed; a high-Arg diet (**HA**), CTL diet plus 0.8% (wt / wt) supplemental L-arginine HCl (SAFC Supply Solutions, St. Louis MO); or a high Arg and vitamin diet (**AEC**), the HA diet plus 200 IU α-

tochopherol / kg of feed (Producers Cooperative Association, Bryan, TX) and 500 mg of ascorbic acid / Kg of feed (Rovimix STAY-C 35, DSM Nutritional Products Inc., Freeport, TX). The diets were iso-caloric and iso-nitrogenous and formulated to meet or exceed all of the NRC (1994) requirements. The chicks were brooded conventionally with temperature starting at 32° C and decreasing 2° C each week until week 3, under a constant lighting program. The chicks were housed in battery cages under normoxic conditions for 14 d.

Extra-Pulmonary Bronchus Occlusion

At 14 d of age, experimental chickens from the dietary groups (i.e. CTL, HA and AEC) were randomly allocated to a control (SHAM) group or to a primary bronchus occlusion group (PBO), as follows. Forty chickens from each dietary group (i.e. CTL, HA and AEC) were assigned to PBO surgery group and an additional 40 birds from the CTL group were assigned to the SHAM surgery group. The surgical procedure was performed as described by Wideman et al. (1996). In short, birds were anesthetized to a surgical plane with intramuscular injections of a 1:1 mixture of ketamine HCl (Ketaset 100 mg/mL; Wyeth Animal Health, Guelph, ON, Canada) and xylazine (AnaSed 100 mg/ml, Akorn Inc., Decatur, IL), at a dose of 0.01 ml of the mix / 100 g BW (Harvey et al., 1985). Anesthetized chickens were fastened in a supine position with the neck extended. Feathers of the thoracic inlet were removed, and the skin was swabed with Betadine (Purdue Prducts L.P., Stamford, CT). Lidocaine (2 %) was infiltrated intracutaneously along the midline of the thoracic inlet as a supplemental local anesthetic. A midline incision was made, the crop was retracted, and the left extra-pulmonary bronchus was located and clamped (completely occluded) with a silver vascular clip fashioned from 0.50 mm diameter silver wire (World Precision Instruments, Sarasota, Fl). The incision was closed with stainless steel surgical wound clips, and sprayed with a topical antibacterial powder. The chicks were placed under a

heat lamp for up to two hours to recover from anesthesia, then they were returned to their cages.

Pulmonary Artery Collection

At the end of the experiment, 28- to 35-day-old the chickens were euthanized. After confirming that the clamp was in place, the heart and lungs were separated, the pulmonary artery devoid of surrounding fat, was placed in a 50-ml plastic tube with a Krebs–Henseleit solution (KHB; 119 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$ and 5.6 mM glucose), maintained on ice in bubbled Krebs buffer with a gas mixture (95 % O_2 and 5 % CO_2). Two 2 - 3 mm rings were taken at 2 mm distance from the pulmonary artery bifurcation of each bird.

Pulmonary Artery Mounting

The pulmonary artery rings were mounted as described by Stallone (1994). Extreme care was taken during preparation of the rings to avoid stretching the tissue or touching the luminal surface to preserve the endothelium, which was functionally evaluated in all experiments. The rings were mounted on two 25-gauge stainless steel wires; the lower one was attached to a stationary stainless steel rod and the upper one was attached to an isometric transducer (Grass FT-03D) connected to a PowerLab data acquisition system (ADInstruments, Pty Ltd., Bella Vista, Australia). Immediately after mounting, the artery rings were suspended in water-jacketed organ bath filled with 15 ml of Krebs-Henseleit buffer solution (KHB) at 37.7 °C and continuously gassed with 95 % O_2 and 5 % CO_2.

Determination of Optimal Artery Tension

The optimal passive tension was determined by repeatedly contracting vessels with 80 mM KCl at progressive 0.5 g increments in passive tension, from 0.5 g to 3 g. Before the start of the experiments, the artery rings were stretched gradually (over a 10 min period) to the established optimal passive tension (start here) and then equilibrated for 90 - 120 min. During the equilibration period, the bathing solution in the organ baths was replaced with freshly gassed, warmed Krebs-Henseleit solution changes every 20 min. The optimal passive tension was adjusted (when necessary) throughout the equilibration and experimental periods.

Checking Viability of the Artery Smooth Muscle and Endothelium Before and After the Experiments

After the equilibration period, paired artery rings from each bird were allowed to stabilize by a near-maximal contraction with phenylephrine (PE; 1×10^{-5} M). After the contraction reached a stable plateau tension, the endothelium-dependent vasodilator acetylcholine (Ach) was added to the bath (1×10^{-6} M). After completion of the PE dose response experiments, vessel preparations were tested again for viability by washing out drugs, allowing the vessels to return to baseline tone, and once again constricting with 80 mM KCl.

Dose-Response Curve to Phenylephrine

After checking vessel viability (with PE followed by acetylcholine), the baths were changed twice and the vessels were allowed to re-equilibrate for 30 - 45 min. After the equilibration period, PE was added to the baths in a cumulative manner to obtain a concentration-response curve for each ring, allowing a stable plateau tension to be attained at each concentration. The effect of phenylephrine on vascular reactivity was

assessed as described by Stallone (1994) with modification, as follows. Briefly, for arteries from the HA-PBO group, L-arginine (2.5 mM) was added to the tissue bath Krebs buffer (**HA-PBO+Arg**) of one pulmonary artery ring of each pair during the re-equilibration period (30 - 45 min), before the dose-response experiment. The vehicle control was the Krebs buffer (**ARG-PBO+VehCtl**). For arteries from the AEC-PBO group, the baths were supplemented with L-arginine (2.5 mM), vitamin C (ascorbic acid; 1 mM) and vitamin E (100 μM). In this case the vehicle control buffer consisted of Krebs buffer plus 0.2 % BSA and 0.1 % DMSO. Vitamin E was brought into solution as described by Olson and Seidel (2000), with modifications; briefly, to bring vitamin E ([±]-α-tocopherol; Sigma T-3251) into solution, it was first dissolved in DMSO as a 2000-strength stock, stored in the dark at 4 °C, and then diluted in Krebs buffer containing 0.2 % BSA and 0.05 % DMSO to final concentration of 100 μM VE and 0.1 % DMSO. The concentration of VE (100 μM α -tocopherol) used in this experiment was found effective for in vitro protection of bovine embryos against ROS (Olson and Siedel, 2000). The level of vitamin C used (1 mM; 176 μg / ml) is above the level that showed a maximal cellular production of nitric oxide in human endothelial cell when incubated for 24 h with 100 μM L-ascorbic acid (17.6 μg / ml) (Heller et al., 1999). It is also above the plasma concentration (18 μg / ml) measured in broiler chickens fed supplemented 500 mg / L water (Pardue et al., 1984). The level of L-arginine used in the present experiment was shown to provide a non-limiting amount of substrate for endothelial nitric oxide synthase activity in in vitro studies where rat thoracic aorta rings were exposed to vasopressin (Stallone, 1994). The addition of Arg, VE and VC to the buffer during the re-equilibrium period (30 - 45 min), before the PE dose response experiments, to the baths containing the PA from the supplemented group of birds (i.e. HA-PBO and AEC-PBO) sought to mimic the blood composition in the live birds in terms of Arg, VE and VC content. For CTL-PBO and CTL-SHAM groups, a single artery ring was used and no supplements were added to the tissue bath Krebs buffer.

Determination of Physiological Parameters and Cardiac Morphology of Broiler Chickens

The experimental birds were monitored daily for mortality, the cause of death was determined and the heart was dissected to determine right ventricle weight/total ventricle weight ratio (**RV/TV**) (Burton et al., 1968) The RV/TV ratios were also determined in birds that were humanely killed at the time of PA harvesting. Body weights (**BW**) and hematocrit (% **Hct**) were recorded at the sampling times.

Vacutainers were used to collect 2 mL of blood from 10 clinically healthy birds in each group. The blood was allowed to coagulate for 30 min in the tubes and then they were centrifuged at 1,500 X *g* for 15 min; serum was collected in labeled tubes and stored at −80 °C until analysis. Uric acid was determined following the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI). In this assay, uricase catalyzes the conversion of UA to allantoin, H_2O_2, and CO_2. H_2O_2 in the presence of horseradish peroxidase, reacts with ADPH (10-acetyl-3,7,-dihydroxyphenoxazine) to produce the highly fluorescent compound resorufin. UA dilutions ranging between 0 and 50 μM were used as standards. Resorufin fluorescence was analyzed with an excitation wavelength of 535 nm and an emission wavelength of 590 nm using a plate reader (Biotek Instruments, Inc., Vermont, USA).

Data Analyses

Pulmonary artery reactivity (**PAR**) to PE was normalized by dry weight of pulmonary artery rings and was expressed as milligram tension per milligram ring weight (mg / mg). Data from the dietary treatment groups (CTL-PBO and CTL-SHAM) and from the buffer supplementation groups (HA-PBO+Arg and AEC-PBO+A-E-C, HA-PBO-VehCtl, AEC- PBO-VehCtl) were analyzed using a one-way ANOVA to detect significant differences. Regarding the vasodilation responses to acetylcholine, the resulting tension was expressed as a percentage of the contraction induced by 1 X 10^{-6} M

PE. In the case of the PE-dose response experiment, comparisons among means were conducted at the middle (1 X 10^{-6} M PE) and maximal (1 X 10^{-4} M PE) concentrations of PE.

RESULTS

Determination of the Optimal Pulmonary Artery Tension

The passive versus active tension relationship recorded in pulmonary artery segments from 28 - 35 d broiler chickens is shown in Figure 9. It can be seen that the first inflection point in the curve occurs when the passive force is 1 g, which corresponds to the optimal tension. This was the optimal resting tension that was used in the present study. The lack of difference in optimal tension between arteries from the CTL-SHAM and CTL-PBO groups suggest that the reactive to KCl was not affected by the initial tension.

Effect of Supplemented L-arginine and Vitamins, E and C, on Reactivity of Male Broiler Chicken Pulmonary Artery to Phenylephrine

Chronic occlusion of a primary bronchus accounted for a 12-fold increase in reactivity (measured in mg tension / mg ring weight) at the middle concentration of PE (1 X 10^{-6} M PE; 36.6 ± 8.3 versus 2.9 ± 8.9 mg / mg, for the CTL-PBO versus CTL-SHAM groups, respectively). The difference in reactivity between PBO and SHAM arteries from the CTL group was 10-fold when the vessels were exposed to the maximal concentration of PE (1 X 10^{-4} M PE; 159.3 ± 13.3 mg / mg versus 16.1 ± 14.3 mg /mg for the CTL-PBO versus CTL-SHAM, respectively) (Figure 10). Also, arteries from the HA-PBO+Arg and AEC-PBO+A-E-C birds had a significantly lower ($P < 0.05$) pulmonary artery reactivity (PAR) at the middle concentration of PE, compared to the

CTL-PBO birds (4.9 ± 8.3 mg / mg, 1.0 ± 7.7 mg/ mg and 36.6 ± 8.3 mg / mg, for the HA-PBO+Arg, AEC-PBO+A-E-C and CTL- PBO groups, respectively).

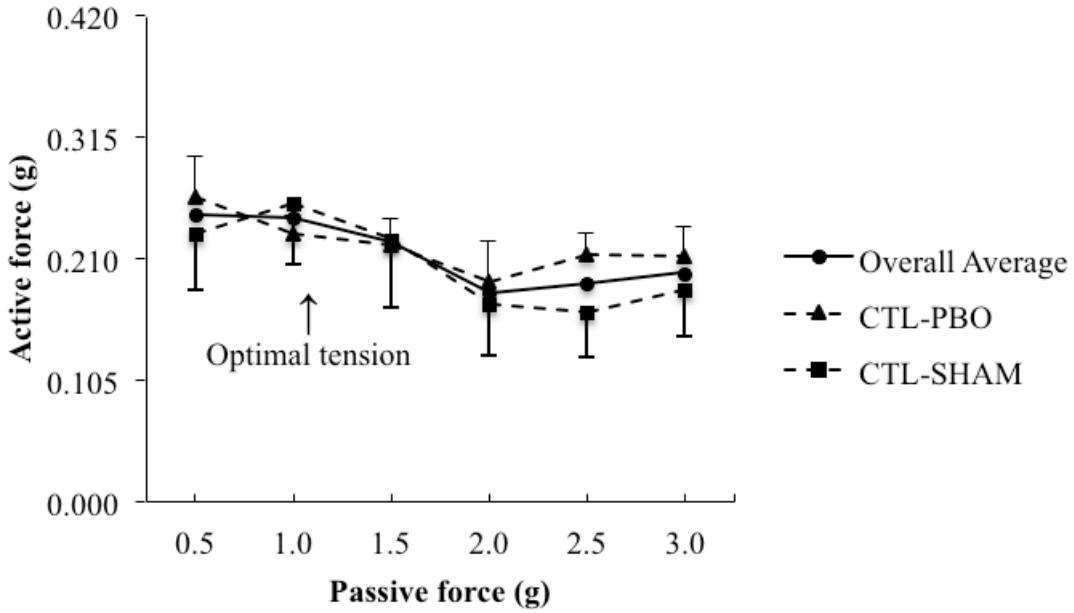


Figure 9**.** Passive versus active force relationship of broiler chicken pulmonary artery rings contracted with 80 mM KCl at 37 °C. The optimal tension is shown by an arrow. CTL = control diet; PBO = primary bronchus occlusion (n=7); SHAM = sham-operated (n=7). Data are expressed as average ± SE.

In addition, the same dietary supplementations significantly ($P < 0.05$) decreased the reactivity of hypoxemic arteries challenged with the maximal concentration of PE (17.9 ± 13.3 mg / mg, 26.1 ± 12.4 mg / mg and 159.3 ± 13.3 mg / mg for the HA-PBO+Arg, AEC-PBO+A-E-C and CTL-PBO, respectively) (Figure 10).

Effect of Arg, VE, and VC Addition to the Bath on Reactivity to Phenylephrine

The addition of Arg (2.5 mM) to the buffer bath of HA-PBO arteries did not have any effect on the reactivity at the middle concentration of PE ($1X10^{-6}$ M; 4.9 ± 8.3 and

4.8 ± 7.7 mg / mg of artery for the HA-PBO+Arg and HA-PBO-VehCtl groups, respectively) or at the maximal concentration of PE (1 X 10^{-4} M; 17.9 ± 13.3 and 16.7 ± 12.4 mg / mg of tissue for the HA-PBO+Arg and HA-PBO-VehCtl groups, respectively).

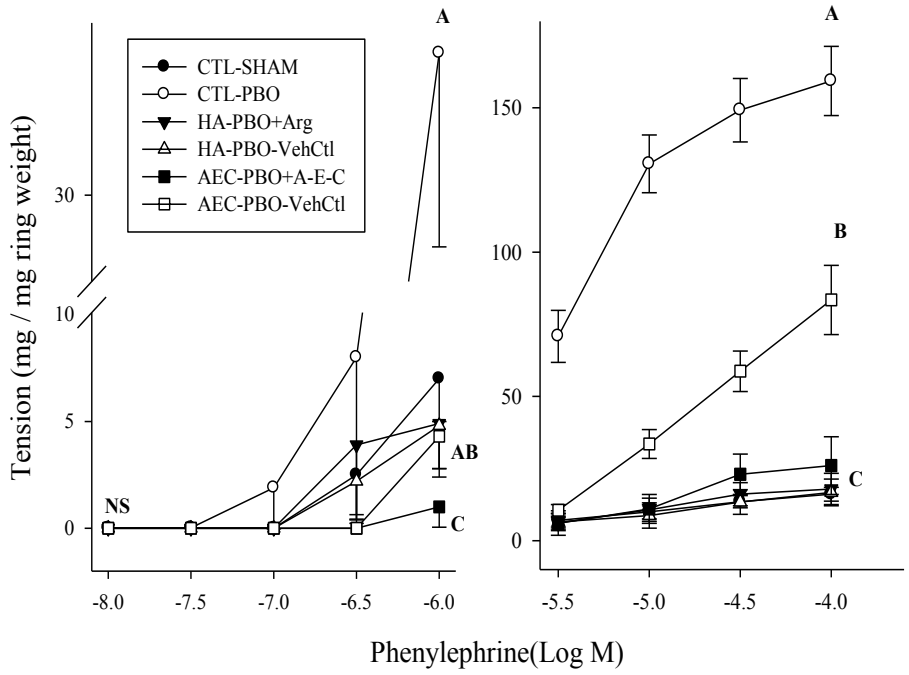


Figure 10. Concentration response curves for phenylephrine (PE) in endothelium-intact pulmonary arteries of male broiler chickens. Left panel: 1 X $10^{-8.0}$ M – 1 X $10^{-6.0}$ M PE; Right panel: 1 X $10^{-5.5}$ M – 1 X $10^{-4.0}$ M PE. Birds were fed either a control un-supplemented diet (CTL; 23% CP, 3200 Kcal/kg, 1.55% arginine, 40 IU vitamin E), a CTL + 0.8% (wt/wt) arginine (HA), or a HA + vitamin E 200 IU/Kg + vitamin C 500 mg/Kg feed (AEC). In addition, at 14 do some birds had either a primary bronchus surgically-occluded (PBO) or were sham-operated (SHAM). CTL-PBO (n=7) and CTL-SHAM (n=6) pulmonary arteries were reacted in buffer. For pulmonary arteries from ARG- PBO birds the experiments were conducted in the presence of either L-arginine (2.5 mM; HA- PBO+Arg; n=7) or its control (buffer; HA- PBO-VehCtl; n=8). For pulmonary arteries from AEC-PBO birds the experiments were conducted in the presence of either L-arginine (2.5 mM) + vitamin E (100 mM)+ vitamin C (1mM; AEC- PBO+A-E-C; n=7), or its control (buffer + 0.2% BSA + 0.1% DMSO; AEC-PBO- VehCtl; n=7). Contractile tension was normalized by dry weight of pulmonary rings. Points represent means ± S.E. ABC represents statistical differences among groups ($P < 0.05$). n=number of birds.

On the other hand, addition of Arg (2.5 mM), VE (100 mM), and VC (1 mM) to the baths of the AEC-PBO arteries reduced the reactivity of the vessels at the middle

(1.0 ± 7.7 mg / mg and 4.3 ± 7.7 mg / mg of tissue for the AEC-PBO+A-E-C and AEC-PBO-VehCtl groups, respectively) or maximal concentration of PE (26.1 ± 12.4 mg / mg and 83.4 ± 12.4 mg / mg of tissue for the AEC-PBO+A-E-C and AEC-PBO-VehCtl groups, respectively) (Figure 10).

Effect of Arg, VE, and VC Addition to the Bath on Reactivity to Acetylcholine and KCl

Before each PE dose-response experiment, the PE-preconstricted (1 X 10^{-5} M) PA segments were tested for viability with the endothelium-dependent vasodilator acetylcholine (Ach; 1 X 10^{-6} M). Acetylcholine produced more relaxation in the PA segments from the HA-PBO than CTL-PBO (Table 4). There were no differences among the rest of treatments in their vasodilatory response to acetylcholine.

Table 4. Contractile responses (mg / mg ring weight) to KCl (80 mM) and % Relaxation response of PE-preconstricted pulmonary arteries to acetylcholine (Ach; 1 X 10^{-6} M) in intact male broiler chickens. Ach was added to the bath before and KCl was added at the end of the PE concentration-response experiments.

Treatment	KCl	% Relaxation
AEC-PBO	161.6 ± 29.4	81.4 ± 17.1
AEC-PBO-VehCtl	142.6 ± 31.4	62.5 ± 17.1
HA-PBO	261.2 ± 27.7	133.8 ± 16.1 [a]
HA-PBO-VehCtl	236.0 ± 27.7	83.1 ± 19.4
CTL-PBO	255.4 ± 27.7	44.9 ± 18.2 [b]
CTL-SHAM	255.9 ± 33.9	59.4 ± 21.0

The birds were fed either a control un-supplemented diet (CTL; 23 % CP, 3200 Kcal/kg, 1.55 % arginine, 40 IU vitamin E), a CTL + 0.8 % (wt/wt) arginine (HA), or a HA + vitamin E 200 IU/Kg + vitamin C 500 mg/Kg feed (AEC). In addition, at 14 do some birds had either a primary bronchus surgically-occluded (PBO) or were sham-operated (SHAM). CTL-POB (n=7) and CTL-SHAM (n=6) pulmonary arteries were reacted in buffer. For pulmonary arteries from ARG-PBO birds the experiments were conducted in the presence of either L-arginine (2.5 mM; HA-PBO+Arg; n=7) or its control (buffer; HA-PBO-VehCtl; n=8). For pulmonary arteries from AEC- PBO birds the experiments were conducted in the presence of either L-arginine (2.5 mM) + vitamin E (100 mM)+ vitamin C (1 mM; AEC- PBO+A-E-C; n=7), or its control (buffer + 0.2 % BSA + 0.1 % DMSO; AEC- PBO- VehCtl; n=7). Contractile tension was normalized by dry weight of pulmonary rings. Means ± S.E. [abc] represents statistical differences among treatments (P < 0.05).

Reactivity of SHAM and PBO pulmonary arteries to 80 mM KCl were obtained following the concentration-response experiments with PE to determine the effects of hypoxemia and diet on PAR. Maximal contractions to 80 mM KCl were higher in the HA-PBO than in the AEC-PBO+A-E-C group; there were no differences among the rest of the treatments (Table 4).

Physiological Parameters and Cardiac Morphology

The % Hct value, a measure of erythropoietic activity to compensate for chronic hypoxemia, was lowest in the CTL-SHAM chickens, with no differences among birds in the other treatments (Table 5). The RV/TV ratio, a measure of right ventricular hypertrophy as a result of sustained high pulmonary hypertension, was lower in the CTL-SHAM birds compared with that of birds in the AEC-PBO group; however, RV/TV ratio was not different among AEC-PBO, HA-PBO, and CTL-PBO groups.

Table 5. Physiological and morphological variables of experimental groups of male broiler chickens.

Treatment	UA (μM)	% Hct	RVW/TVW ratio	BW (g)	% PHS
AEC-PBO	442.5 ± 33	39.4 ± 1.9 [a]	0.25 ± 0.02 [a]	1827 ± 153	7.9 (3/38) [a]
HA- PBO	486.4 ± 33	37.6 ± 1.7 [a]	0.22 ± 0.01 [ab]	1767 ± 120	10.0 (4/40) [a]
CTL- PBO	438.2 ± 39	34.7 ± 1.7 [ab]	0.21 ± 0.02 [ab]	1734 ± 130	7.7 (3/39) [a]
CTL-SHAM	509.7 ± 42	29.6 ± 1.7 [b]	0.17 ± 0.01 [b]	1882 ± 130	2.4 (1/41) [b]

Broilers were fed either a control un-supplemented diet (CTL; 23% CP, 3200 Kcal/kg, 1.55% arginine, 40 IU vitamin E), a CTL + 0.8% (wt/wt) arginine (HA), or a HA + vitamin E 200 IU/Kg + vitamin C 500 mg/Kg feed (AEC). In addition, at 14 do some birds had either a primary bronchus surgically-occluded (PBO) or were sham-operated (SHAM). UA = uric acid content in serum; %Hct = hematocrit percentage (means ± S.E); RVW/TVW ratio = right ventricle weight / total ventricle weight ratio; BW = body weight (means ± S.E); %PHS = pulmonary hypertension syndrome incidence (%). [abc] represents statistical differences among treatments ($P < 0.05$). Number of birds = 6-12.

The PBO-birds had higher mortality than the SHAM birds, and dietary treatment did not reduce mortality due to pulmonary hypertension. There were no differences in BW among the different experimental groups (Table 5). The serum uric acid concentration was not affected by either dietary treatment or PBO.

DISCUSSION

In the present investigation, the effects of supplemental Arg, VC and VE on PAR to a non-peptide vasoconstrictor, the α1-adrenoceptor agonist PE, were examined in pulmonary artery rings from hypoxemic broiler chickens. The results suggest that supplemental Arg and antioxidant vitamins E and C have an important modulatory effect on PAR to PE in hypoxemic broiler chickens. These results are in agreement with previous findings that chronic supplementation with Arg, VE and V**C** lowered pulmonary arterial pressure (PAP) levels faster to basal levels in hypertensive broiler chickens than in unsupplemented controls, after challenges with bolus injection with epinephrine (Epi; Lorenzoni and Ruiz-Feria, 2006; Ruiz-Feria, 2009; Butista-Ortega and Ruiz-Feria, 2010).

Optimal Pulmonary Artery Tension

In the present study, the optimal passive tension was determined to be 1 g in the pulmonary arteries from clinically healthy hypoxemic broiler chickens and this value was lower than the 2 g reported by Alvarez-Medina et al. (2011) for broiler chickens. This difference may be partially explained by the fact that in the present investigation the organ baths were warmed to 37.7 °C while Alvarez-Medina et al. (2011) conducted the experiments at 42 °C. In this context, in the present investigation, it was observed that mounted pulmonary arteries that had been kept on ice and then bathed in warmed Krebs buffer (during mounting) contracted immediately.

Effect of Supplemented L-arginine and Vitamins, E and C, on Reactivity of Male Broiler chicken Pulmonary Artery to Phenylephrine

Pulmonary hypertension syndrome is associated with morphological and functional changes in resistance pulmonary arteries, mainly hypertrophy and hyperplasia of smooth muscle, and altered vascular reactivity and Ca^{+2} homeostasis (Shimoda et al., 2000; Rabinovitch, 2007). Functional alterations have been noted in the pulmonary artery of hypoxic chickens (Odom, et al., 2004; Alvarez-Medina et al., 2011) and mammals (Shimoda et al., 2000) indicating that the pulmonary artery can be used as a relevant model for the study of pulmonary vasoreactivity in chronic hypoxia or hypoxemic conditions.

In the pulmonary artery from chickens endothelium-dependent vasodilation is mediated by nitric oxide (Odom et al., 2004) and endothelial derived hyperpolarizing factor (**EDHF**) (Alvarez-Medina, et al. 2011) or both; whereas prostacyclin (**PGI_2**) is not an effective pulmonary vasodilator (Wideman et al., 2005). More specifically, NO was shown to account for almost 100 % of the relaxation induced by acetylcholine in endothelium-intact pulmonary arteries of non-hypertensive (RV/TV ratio = 0.21) broiler chickens (Alvarez-Medina et al., 2011). However in endothelium-intact pulmonary arteries of hypertensive broiler chickens (RV/TV ratio = 0.43) EDHF accounted for 83% of the vasodilation induced by acetylcholine (Alvarez-Medina et al., 2011). In the present investigation we used clinically healthy broiler chickens (RV/TV ratio = 0.17 to 0.25; Table 5) and so the endothelium-dependent vasodilation induced in endothelium-intact pulmonary arteries by either 1 X 10^{-6} M acetylcholine or as a response to counteract the contraction induced by PE most likely came from NO.

In the present study chronic hypoxemia (created by occluding one primary bronchus) caused pulmonary artery hyperreactivity (contraction) to PE in the CTL-PBO group of broiler chickens compared to the CTL-SHAM one. This result is consistent with the enhanced contraction responses to endothelin-1 and angiotensin-II documented in mammalian models of chronic hypoxic pulmonary hypertension (Shimoda et al.,

2000). Taken together, these findings suggest that chronic hypoxemia and hypoxia induce changes downstream the contraction receptors of the pulmonary artery smooth muscle. The most widely accepted mechanism through which hypoxia causes increased reactivity to constricting substances in the pulmonary artery involves ROS and increased Ca^{2+} sensitization. Ca^{2+} sensitization has been defined as the process by which a stimulus causes an increase in contractile force without the necessity for an increase in intracellular Ca^{2+} concentration (Ward and Knock, 2011).

In this regard, it has been reported that exposure to chronic hypoxia increased ROS generation and endothelin-1-induced cytosolic Ca^{2+} sensitization, which correlated with Rho kinase-dependent myosin phosphate phosphorylation (Jernigan et al., 2008). In the same way, in rat small pulmonary artery, superoxide triggered Rho-kinase-mediated cytosolic Ca^{2+} sensitization and vasoconstriction (Knock et al., 2009). This pathway can be summarized as follows: when RhoA kinase (a small monomeric G protein) is activated by ROS, it phosphorylates myosin phosphatase target protein subunit 1 (MYPT1), which inhibits myosin light chain phosphatase (MLCP), so myosin light chain (MLC) remains phosphorylated, which enhances calcium sensitization (Mishra et al., 2011). Basal or resting levels of cytosolic Ca^{2+}concentration are sufficient to support the RhoA kinase-mediated Ca^{2+} sensitization and contractile force (Ward and Knock, 2011).

Because the onset of PHS and IPAH coincide with a generalized increased production of ROS (Enkvetchakul et al., 1993; Wedgwood and Black, 2003; Pan et al., 2007; Nain et al., 2008a,b), in the present investigation Arg, VC and VE were supplemented chronically before and during the PBO challenge, which is known to induce PHS. It was expected that these supplements improved pulmonary artery reactivity (in vitro) to PE in agreement with an improved cardiovascular performance in an in vivo chicken model of PHS. In agreement with the findings in the in vivo chicken model of PHS, it was found that pulmonary artery segments from supplemented chickens (HA-PBO-VehCtl) showed striking reduced reactivity to PE compared to those from the CTL-PBO group of chickens (Figure 10), suggesting a protecting role of Arg against chronic hypoxemia-induced hyperreactivity. In addition, the reactivity to PE in

pulmonary arteries from the two Arg-supplemented groups was reduced to levels similar to those seen in the pulmonary artery segments from the SHAM group (CTL-SHAM; Figure 10). The most widely accepted protective mechanism of chronic supplemental Arg in the PHS scenario includes boosting substrate availability for eNOS to enhance NO production (Carville et al., 1993; Wideman et al., 1995); in turn, NO has been shown to quickly react with superoxide to produce peroxynitrite at the expense of NO bioavailability in PHS (Shuvaev et al., 2009).

However, in the present investigation concurrent supplementation with Arg plus VE and VC did not show an apparent further decrease in reactivity to PE in pulmonary artery rings from the AEC-supplemented birds (AEC-PBO-VehCtl; Figure 10). This result was unexpected and in disagreement with the in vivo chicken model of PHS. In other words, chronic concurrent supplementation with Arg plus VE and VC was expected (1) to increase production of NO by the added Arg, and (1) to neutralize ROS thus increasing the bioavailability of the produced NO. Thus a better pulmonary artery reactivity was expected when feeding supplemental Arg and antioxidant vitamins than that obtained by feeding supplemental Arg alone. A possible explanation is a follows. It was observed that pulmonary artery rings from the CTL-PBO broilers tended to spontaneously contract during the stabilization period while those from the HA-PBO (to a lesser degree) and AEC-PBO (to a higher degree) birds tended to relax compared to pulmonary artery rings from the CTL-SHAM group of birds. In either case adjustments were necessary in order to maintain the optimal tension at 1 g. The fact that AEC-PBO vessels relaxed at a higher degree probably led to an overestimation of the reactivity of theses vessels to the PE concentrations studied. In other words, AEC may have been less reactive to PE than actually reported above (i.e. the dose response curve in the AEC-PBO groups should actually be shifted downwards) and certainly less reactive to PE than HA-PBO vessels.

Effect of Bath Supplementation on Pulmonary Artery Reactivity to Phenylephrine

In order to further investigate the effects of Arg and antioxidant vitamins E and C, on the reactivity of pulmonary arteries to PE, Arg, VE and VC were added to the buffer (and hence to tissue baths) during the re-equilibration period (30 - 45 min) following the acetylcholine challenge in PE-preconstricted arteries and before the PE dose-response experiments. Final supplement concentrations in the buffer used in the present study have been shown to be well above the concentrations that protect embryos against oxidative stress (VE), maximize NO production by the endothelial cells (VC) and that provide non limiting substrate (Arg) availability for eNOS to produce NO in vitro (Stallone, 1994; Heller et al., 1999; Olson and Siedel, 2000).

In this context, results of the present study showed that preincubation with Arg did not further reduce the PA reactivity to PE in the HA-PBO+Arg artery segments most likely because Arg, substrate for eNOS, was not a limiting factor in the pathway that leads to the production of NO (i.e. there were no differences between the HA-PBO+Arg and HA-PBO-VehCtl groups, Figure 10). In partial agreement with this result, Odom et al. (2004) found that under acute hypoxia under in vitro conditions L-Arg (1×10^{-4} M) supplementation even reduced acetylcholine-induced vasodilation in pulmonary artery of broilers raised under normoxic conditions. The reasons for the discrepancies in the results obtained in the present study and those reported by Odom et al. (2004) on the effect of in vitro supplemental Arg may include: (1) the present study used pulmonary arteries from birds raised under chronic hypoxia, (2) the in vitro experiment was conducted under normoxic conditions, and (3) not to mention differences in the concentration of Arg used. Taken together these results indicate that compared to an in vivo model of PHS there may be differences not only in the uptake of Arg when compared to an experimental in vitro set up but also in the level of hypoxia to which the arteries are exposed both in vitro and in vivo (Carville et al., 1993; Odom et al., 2004).

In addition, results from the present study showed that when PA segments from the AEC-PBO group (dietary supplemented with Arg + VE + C) of chickens were

preincubated (before the PE dose response experiment) with Arg (2.5 mM) plus VE (100 mM) and VC (1 mM) (AEC-PBO+A-E-C), their reactivity to PE decreased compared with that of the PA of the AEC-PBO-VehCtl groups. More specifically, the combined effect of Arg, VE and VC in the bath reduced more than 4-fold the reactivity to middle (1×10^{-6} M) and maximal concentration of PE (1×10^{-4} M). This result is in agreement with the notion that ROS may be involved in endothelial dysfunction (e.g. increasing reactivity to PE) in the hypoxemic pulmonary wall, as mentioned above. In this context, it is possible that in the present study the antioxidant vitamins, E and C, reduced ROS and thus downregulated the Rho/ROK pathway leading to a lesser contraction in the AEC-PBO+A-E-C than in the AEC-PBO-VehCtl pulmonary arteries.

In the present study pulmonary arteries from both normoxic and hypoxemic un-supplemented broiler chickens reacted to PE in the concentration range between 1×10^{-8} M to 1×10^{-4} M thus agreeing with the results presented by Alvarez-Medina et al. (2011) who conducted research on non-hypertensive (RV/TV ratio = 0.21) and high-altitude-induced hypoxic (hypobaric hypoxia) broiler chickens (RV/TV ratio = 0.43). However, a direct comparison regarding the degree (size) of the response to PE in both groups of birds obtained in the present study with those by these authors is not possible because the authors presented their results on the contraction induced by PE as a percentage of a maximal contraction induced by 40 mM KCl.

Reactivity of Pulmonary Artery to Acetylcholine and KCl

In the present investigation, acetylcholine was added to the organ bath of PE-preconstricted pulmonary arteries before the PE-dose response experiments. A pulmonary artery vasodilation response was evidence of an intact endothelium. Acetylcholine causes endothelium-dependent vasodilation and in the case of the clinically healthy chicken pulmonary artery, NO is the main vasodilator (Zoer et al., 2009; Odom et al., 2004). In the present investigation, acetylcholine induced a higher

vasodilation response in the HA-PBO group than in the CTL-PBO; whereas there were no differences among the rest of the treatment groups (Table 4).

Potassium chloride depolarizes smooth muscle by shifting the K^+ equilibrium to the left. In this regard, KCl constitutes a receptor-independent stimulus used in preparations in vitro to study the possible interacting effects of reagents on contractility when compared to PE-induced (receptor mediated) contraction (Billaud et al., 2011). Supplementation with Arg or Arg plus VE and VC did not affect pulmonary artery reactivity to KCl (Table 4). Accordingly, these results suggest that hyperreactivity to PE seems to be receptor dependent.

Physiological Parameters and Cardiac Morphology

The RV/TV ratio is commonly used as a measured of the hypertension level, which directly causes right ventricular hypertrophy in broiler chickens. Clinically healthy domestic fowl with normal pulmonary arterial pressure have RV/TV ratios ranging from 0.15 to 0.27, whereas sustained pulmonary hypertension causes RV/TV ratios above 0.28 (Wideman, 2001).

The overall RV/TV ratio in the hypoxemic (PAO) broiler chickens was 0.23 ± 0.02 while that of the normoxic ones (SHAM) was 0.15 ± 0.01 (Table 5), indicating that all the experimental birds were clinically healthy.

In general the PBO groups of birds were more hypertensive and more hypoxemic, as result of higher polycythemia (increased % Hct values), than the normoxic ones (SHAM) (Table 5). As a consequence hypoxemic chickens presented higher PHS incidence than the normoxic ones. These results attest to the effectiveness of the primary bronchus occlusion to induce PHS in broiler chickens. The fact that these physiological parameters were in general similar among the hypoxemic groups suggest that the differential reactivity responses observed in the pulmonary arteries to PE were brought about by the chronic supplementation of Arg or Arg plus VE and VC.

Body weight did not differ significantly among the experimental groups suggesting that this parameter was not affected by hypoxemia or supplements in the diet (Table 5). Body weight, specially the rate of growth, is a factor in PHS whereby fast-growing broilers are more likely to develop the condition. In this regard, in the present investigation growth rate was not a confounding variable.

In birds, uric acid (UA) is formed as an end-product of both purine metabolism and the deamination of that occurs in protein catabolism (Stevens, 1996). Chickens and humans lack the enzyme uricase, which converts UA into allantoin for excretion. It has been reported that there is a negative relationship between plasma / serum UA concentrations and oxidative stress in the chicken (Carro et al., 2010). The UA concentration determined in the serum from the experimental groups is shown in Table 5. Supplementation with Arg, VE and VC did not affect the UA concentration in the serum of the experiment groups of birds. This may suggest that UA did not function as an antioxidant to an extent as to show a significant reduction in the concentration of this metabolite in the serum from the experimental birds. This result may further indicate that UA was not oxidized (non-enzymatically) into allantoin by ROS present in the experimental birds. Our results differ from those presented by Carro et al. (2010) because these authors used allopurinol to inhibit xanthine oxidase leading to reduction in UA production and a concomitant increase in ROS production; ROS non-enzymatically oxidase UA to allantoin.

In conclusion, the present studies demonstrate that PE-induced contraction in the pulmonary artery segments is greater in hypoxemic unsupplemented broiler chickens than in normoxic unsupplemented ones. Chronic supplementation with Arg or chronic concurrent supplementation with Arg plus VE and VC reduced the reactivity to PE in hypoxemic chickens. These differential responses in PA reactivity to PE seem to be brought about by protective effects of the chronic supplementation and imply an important role of ROS in the observed endothelial dysfunction as well in the upregulation of smooth muscle contractility.

CHAPTER VI

PULMONARY VASCULAR REMODELING IN BROILER AND LEGHORN CHICKENS AFTER UNILATERAL PULMONARY ARTERY OCCLUSION

INTRODUCTION

Unilateral pulmonary artery occlusion (**PAO**) is a surgical procedure that places a large workload on the right ventricle of the heart (Wideman and Kirby, 1995a, 1995b). By occluding one pulmonary artery the blood volume perfusing the unobstructed lung (pulmonary artery) is doubled. Thus, a proportionally high cardiac output together with a low capacity non-compliant pulmonary vasculature can lead to a ventilation/perfusion mismatch, hypoxaemia and to a rapid development of pulmonary hypertension syndrome (**PHS**) in fast-growing broilers (Nagasaka et al., 1984; Koyama and Horimoto, 1993; Reeves and Rubin, 1998). Wideman and Kirby (1995b) reported that PAO induced pulmonary hypertension, with dilated hearts as early as 24 h after PAO. In general, PAO initiates a progression of symptoms typical of those observed in broilers developing PHS spontaneously, under a variety of environmental and commercial conditions, or during exposure to cold or hypobaric hypoxia. Leghorn chickens, however, have been reported to be resistant to PHS and to show superior cardiovascular capacity (Hassanzadeh et al., 2005) and better endothelium-dependent vasodilation (Martinez-Lemus et al., 1999) under normoxia compared to broiler chickens.

In susceptible fast-growing broiler chickens small diameter arterioles are responsible for the increased resistance to blood flow in the lungs. For example, hypoxic vasoconstriction resides proximal to the capillaries and occurs in resistance arterioles 30 to 300 μm in diameter (Reeves and Rubin, 1998). Endothelial dysfunction and smooth muscle hypertrophy or proliferation (remodeling) are key histopathological changes of PHS in animal models (Wideman et al., 2011) and in idiopathic pulmonary arterial hypertension (**IPAH**) in humans (Wagenvoort and Wagenvoort, 1970). The red jungle

fowl, ancestor of both broilers and Leghorns, showed a transient increase in pulmonary arterial pressure when subjected to acute PAO (i.e. acutely tightening a snare around the pulmonary artery) followed by flow-dependent vasodilation, which brought pulmonary arterial pressure back to basal levels (Wideman et al., 1998). The red jungle fowl has superior cardiovascular capacity than Leghorn chickens, and Leghorns have a better cardiovascular capacity than that of broilers. These differences in cardiovascular capacity probably reflect physiological changes brought about by the selection pressure placed upon broilers and Leghorns for higher production. The remodeling (e.g. increased medial thickness) of resistance pulmonary arteries (RPA) caused by PAO in the two types of chickens, broilers versus Leghorns, has not been documented. In vitro studies have shown that pulmonary arteries from broiler chickens have a reduced endothelium-dependent relaxation compared to those from Leghorn chickens of the same age (Martinez-Lemus et al., 1999; Odom et al. 2004). In addition, in vitro acute hypoxia demonstrated a higher contraction of intrapulmonary arteries from broiler chickens than in those from Leghorns (Zoer et al., 2009). In the present study male broiler and Leghorn chickens of similar BW and fed a similar diet (a typical high energy broiler diet), were subjected to PAO, in order to conduct a comparison of their physiological responses to chronically increased pulmonary vascular resistance (**PVR**) as a result of the entire cardiac output perfusing a single lung vasculature. We hypothesized that Leghorns will show a similar degree of hypoxemia than broilers chickens, but will not become hypertensive and will avoid PRA vessel remodeling.

MATERIALS AND METHODS

Experimental Design

One d-old male Cobb 500 broiler chicks (n=80) and white Leghorn (Hy-Line) chicks (n=80) were used. The chicks were wing-banded and raised in wire battery cages; they were brooded conventionally with temperature starting at 32° C and

decreasing 2° C each week until they reached the target BW of 800 g, recommended to perform PAO surgery in broiler chickens (Wideman and Kirby, 1995a). Leghorn chickens were given a 6-wk head start growing period to attain a similar BW as broilers. Throughout the experiment, all birds were fed a corn-soybean meal based diet (23% CP, 3200 Kcal/kg), formulated to meet or exceed the requirements for broilers specified by the NRC (1994). When broilers were 18-21 d old and leghorns 61-64 d old, forty birds per strain were subjected to PAO, as described below, whereas another 40 birds per strain were sham-operated. In summary, the experimental treatments were: L-PAO, Leghorns subjected to PAO; L-SHAM, Leghorns sham-operated; B-PAO, broilers subjected to PAO; and, B-SHAM, broilers sham-operated.

Surgical Procedure for Unilateral Pulmonary Artery Occlusion

The surgical procedure for clamping the left pulmonary artery to induce pulmonary hypertension has been previously described (Ruiz-Feria et al., 1999). Birds from the PAO groups (broilers and Leghorns) were anesthetized to a surgical plane with intramuscular injections of a 1:1 mixture of ketamine HCl (100 mg/mL, Bioniche Pharma USA LLC, Lake Forest IL) and xylazine (AnaSed 100 mg/ml, Akorn Inc., Decatur, IL), at a dose of 0.007 to 0.015 ml of the mixture / 100 g BW (Harvey et al., 1985). Anesthetized chickens were fastened in a supine position with the neck extended. Feathers of the thoracic inlet were plucked, and the skin was swabbed with Betadine (Purdue Products L.P., Stamford, CT). Lidocaine (2% s.c. xylocaine, Astra-Zeneca, Wilmington, DE) was infiltrated subcutaneously along the midline of the thoracic inlet as a supplemental local anesthetic. A midline incision was made, the crop and trachea awere retracted to the right, and the left thoracic air sac was opened. The left pulmonary artery was located and clamped with a silver vascular clip fashioned from 0.38 mm diameter silver wire (World Precision Instruments, Sarasota, FL). The incision was closed with stainless steel surgical wound clips, and sprayed with a topical antibacterial powder. The chicks were placed under a heat lamp for up to two hours to recover from

anesthesia, then they were returned to their cages. Another group of birds (broilers and Leghorns) were sham operated; they underwent surgery as described above, the pulmonary artery was isolated but was left intac (not clamped).

Determination of Physiological and Morphological Parameters

Physiological and morphological assessments were made at 3 sampling times from at least 6 randomly chosen chickens per treatment: pre-surgery (1 d before surgery), 7, and 14 days post-surgery. The ventilation capacity was assessed by determining the relative lung weight: total wet lung weight (right lung + left lung) divided by BW and multiplied by 100 (Hassanzadeh et al., 2005). The experimental birds were monitored daily for mortality, the cause of death was determined and the heart was dissected to determine right ventricle weight/total ventricle weight ratio (**RV/TV**) (Burton et al., 1968). The RV/TV ratios were also determined in birds that were humanely killed at the sampling times previously described. The BW and hematocrit (%, **Hc**) were recorded at the sampling times.

Determination of Small Pulmonary Artery Hypertrophy

The right lung (perfused) was excised from six randomly selected chickens from each experimental group. One transverse section of the paleopulmonic region was collected in tubes containing Trump's Fixative. Ten μm-thick sections were cut and subsequently stained with Humberstone and Gomori's elastin stain for elastic tissue. Measurements of medial thickness of at least 20 small muscular pulmonary arteries and arterioles, external diameter 30-200 μm were analyzed per bird. Medial thickness (MT) was estimated as the mean of four measurements for each vessel (MT1 + MT2 + MT3 + MT4) whereas the external diameter (D) was taken as the mean of two measurements at right angles to each other (D1 + D2). Medial thickness was expressed as percentage of external diameter (**% Thickness**): (MT1 + MT2 + MT3 + MT4) / (D1 + D2) X 100

(Tucker et al., 1975). Medial thickness, a measure of vascular remodeling, has been used reliably for estimating the amount of vascular smooth muscle in several species (Tucker et al., 1975). The resistance pulmonary arteries were divided into two groups, 30-100 μm and 100-200 μm diameter, to better assess vascular remodeling. Pictures were taken using a Zeiss digital axioplan microscope using a Zeiss Plan-Apochromat 10X/0.45na objective. The digitalized images were captured by computer and analyzed using the software Image J.

RESULTS

Pulmonary Resistance Artery (PRA) Hypertrophy

The mean diameter of the resistance vessels of less than 100 μm in diameter studied was 70 ± 6 μm, with no differences in mean external diameter among artery groups at any of the three sampling times (Table 6).

Table 6. Average diameter (±SE) of pulmonary arteries in experimental chickens. Male broiler chickens were subjected to surgery to occlude one pulmonary artery (B-PAO) or were sham-operated (B-SHAM), and Leghorn male chickens had an occluded pulmonary artery (L-PAO) or were sham-operated controls (L-SHAM).

		Treatment			
Diameter (μm)	Sampling time	B-SHAM	B-PAO	L-SHAM	L-PAO
<100	Pre-surgery	73 ± 5	73 ± 5	70 ± 5	70 ± 5
	7dPS	66 ± 4	66 ± 4	63 ± 4	73 ± 4
	14dPS	70 ± 10	71 ± 10	63 ± 11	86 ± 11
100-200	Pre-surgery	138 ± 6	138 ± 6	131 ± 6	131 ± 6
	7dPS	133 ± 6	133± 8	130 ± 7	137 ± 6
	14dPS	138 ± 8	134 ± 8	135 ± 9	151 ± 12

7dPS = 7 days post-surgery; 14dPS = 14 days post-surgery. N=40.

Results on % Thickness of PRA are presented in Figure 11. Before surgery there was no difference in PRA thickness among birds of the different groups. At 7 d post-surgery, however, B-PAO (21 ± 1 μm) chickens had thicker ($P < 0.05$) RPA than those from chickens in the other groups; with no differences among the other groups. At 14 d post-surgery, the B-PAO chickens (21.6 ± 0.7 μm; $P < 0.05$) had thicker RPA than the B-SHAM chickens (20.1 ± 0.7 μm). In turn, the B-SHAM group had thicker RPA than both, the L-SHAM (17.6 ± 0.8 μm) and the L-PAO (17.4 ± 0.8 μm) groups, with no differences between the two Leghorn groups.

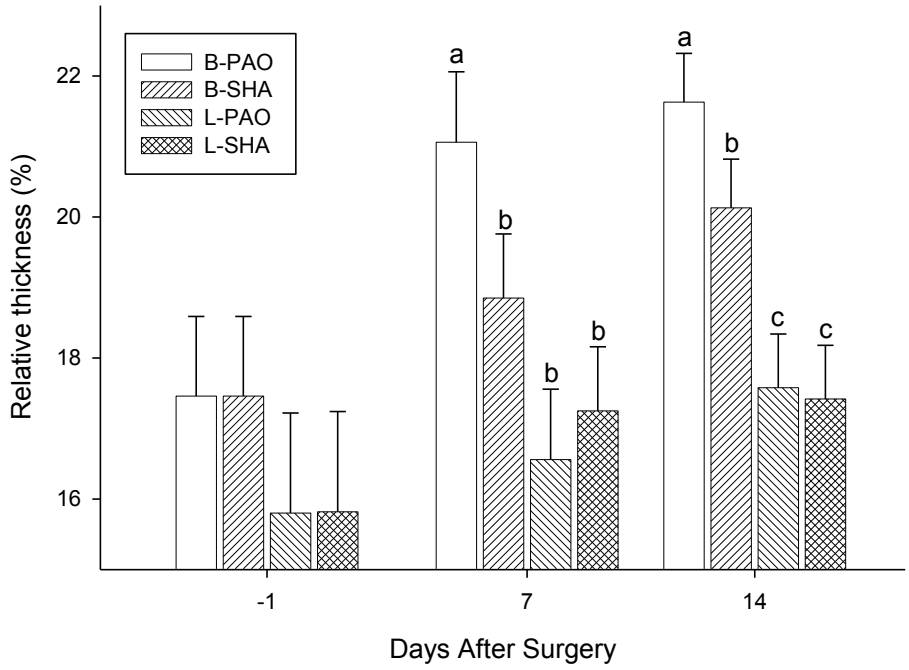


Figure 11. Effect of chicken strain and oxygen level on percentage thickness of pulmonary arteries with <100 μm diameter. Male broiler chickens with one surgically occluded pulmonary artery (B-PAO) and their sham-operated control (B-SHA), and male Leghorns with occluded pulmonary artery (L-PAO) and their control (L-SHA). The thickness of the media (smooth muscle) was divided by the artery diameter and then multiplied by 100. Data are expressed as means ± SE (n = 40 per group and time point); Means within each sampling point without a common letter are different ($P \leq 0.05$).

The mean diameter of the RPA between the diameter 100 -200 μm studied was 138 ± 6 μm. There were no differences in mean external diameter among arteries groups at any of the three sampling times (Table 6).

Before surgery, broilers (16.5 ± 0.7 μm) had thicker PRA than Leghorns (14.0 ± 0.7 μm). At 7 d postsurgery, the B-SHAM group (17.1 ± 0.9 μm) had thicker PRA compared with birds in the other groups, but with no differences among B-PAO, L-SHAM and L-PAO groups of arteries. At d 14 postsurgery, the B-PAO group had ($P <$ 0.05) thicker PRA than the L-PAO group (14.8 ± 1.0 μm); but not different than those of the B-SAHM or L-SHAM birds (Figure 12).

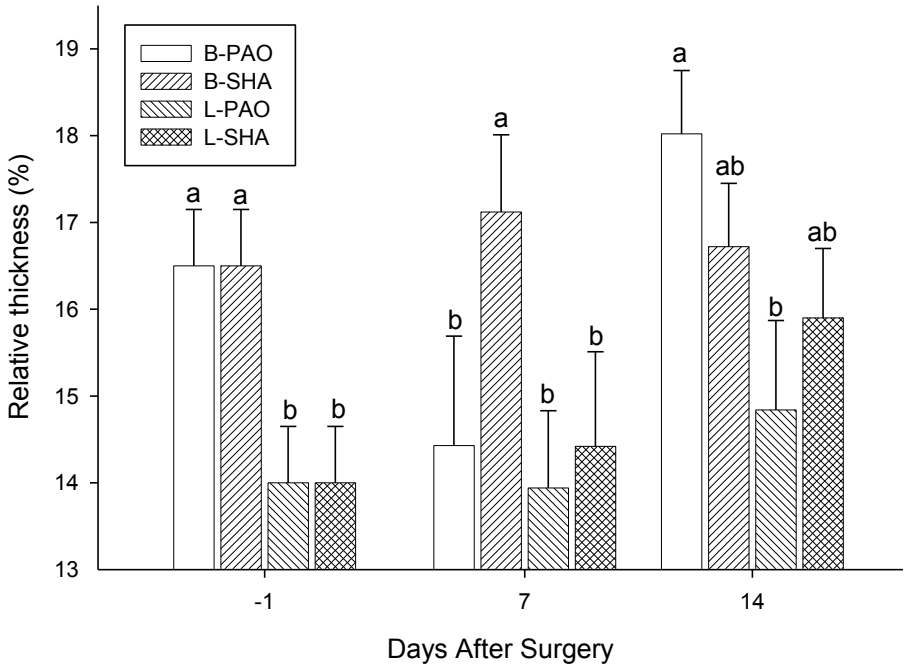


Figure 12. Effect of chicken strain and oxygen level on percentage medial thickness of pulmonary arteries with 100 - 200 μm diameter. Male broiler chickens with one surgically occluded pulmonary artery (B-PAO) and their sham-operated control (B-SHA), and male Leghorns with occluded pulmonary artery (L-PAO) and their control (L-SHA). The thickness of the media (smooth muscle) was divided by the artery diameter and then multiplied by 100. Data are expressed as means ± SE (n = 40 per group and time point). Means within each sampling point without a common letter are different (P ≤ 0.05).

Physiological and Morphological Parameters

There were no differences in relative lung weight between L-SHAM and L-PAO birds, or between B-SHAM and B-PAO birds. Therefore, the data on relative lung weight were pooled by strain of bird. Leghorns had a higher ($P < 0.05$) relative lung weight than broilers at all sampling times. Also, the relative lung weight tended to decrease with age in both types of birds (Figure 13).

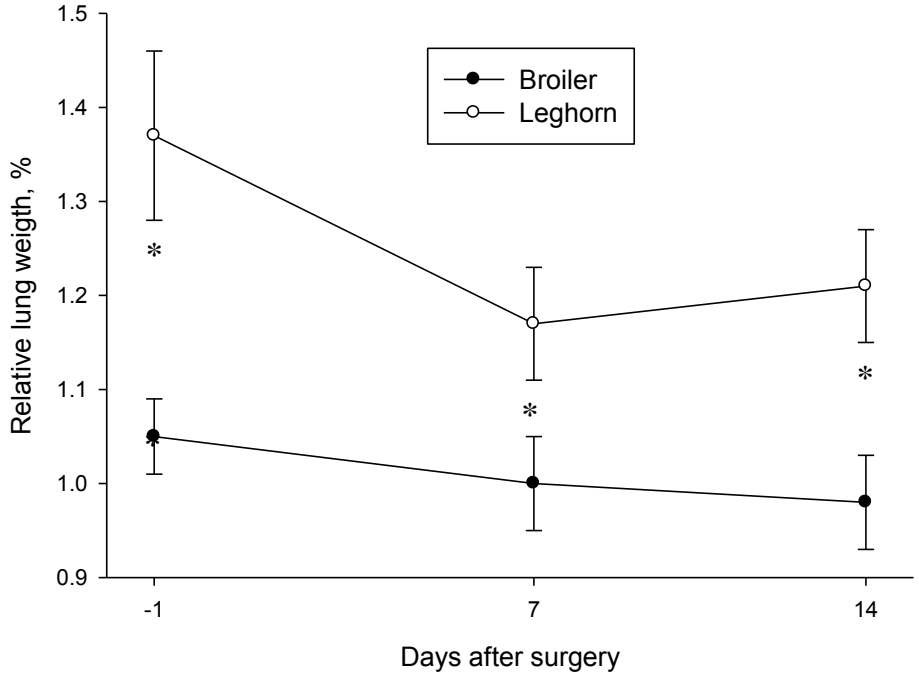


Figure 13. Specific lung weight of male broilers and Leghorns at the different sampling points. The specific lung weight was determined dividing the total lung wet weight by 100 g body weight. Data are expressed as means ± SE (n = 8 per group and time point). * Indicates that means within each sampling are different ($P \leq 0.05$).

Results on hematocrit (%, Hc) are presented in Figure 14. Before surgery, there was no difference among chickens from the different experimental groups. At 7 d post

surgery, the B-PAO group had higher Hc (32 ± 1.2; $P < 0.05$) than the B-SHAM birds (28.4 ± 1.2 %), and the L-PAO group had higher Hc (34.9 ± 1.4; $P < 0.05$) than the L-SHAM birds (31.9 ± 1.2). Also, L-PAO birds had higher Hc than B-PAO birds, whereas L-SHAM and B-PAO birds had similar Hc levels.

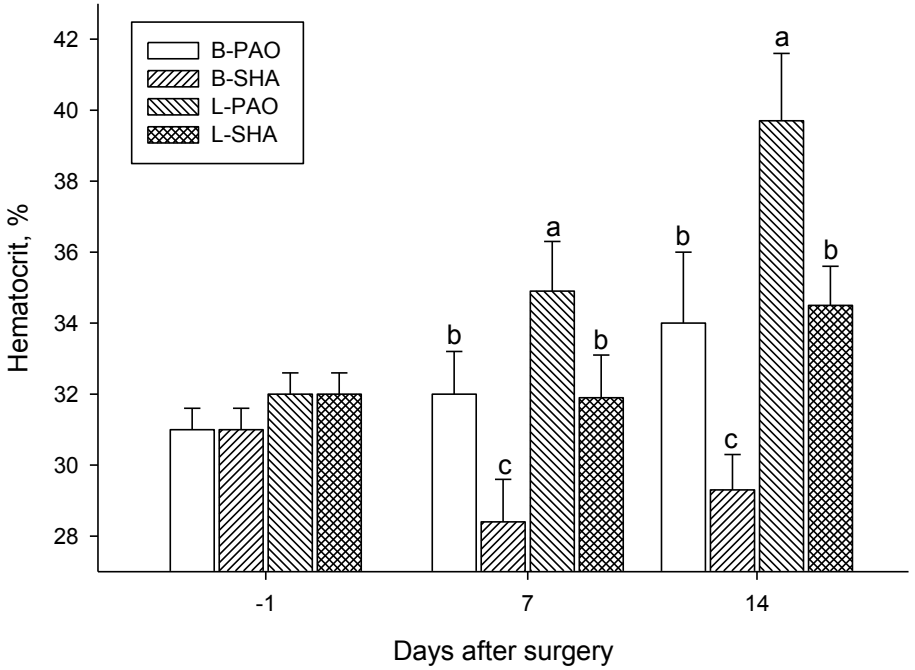


Figure 14. Effect of chicken strain and oxygen level on hematocrit. Chickens with one surgically occluded pulmonary artery (B-PAO) and their sham-operated control (B-SHAM), and male Leghorns with occluded pulmonary artery (L-PAO) and their control (L-SHAM). Data are expressed as means ± SE (n = 8 per group and time point). Means within each sampling point without a common letter are different ($P \leq 0.05$).

At 14 d post surgery, the B-PAO birds had higher Hc (33.9 ± 2.0; $P < 0.05$) than the B-SHAM birds (29.3 ± 1.0), and the L-PAO birds had higher Hc (39.7 ± 1.9) than the L-SHAM birds (34.5 ± 1.1). Also, L-PAO birds had higher Hc than B-PAO birds, whereas L-SHAM and B-PAO birds had similar Hc values. Results on RV:TV ratio are presented in Figure 15. Before surgery, broiler chickens (0.16 ± 0.01) had lower RV:TV ratios than Leghorns (0.19 ± 0.01; $P < 0.05$). At 7 (0.31 ± 0.06) and 14 d (0.32 ± 0.03)

post surgery B-PAO chickens had the highest RV:TV ratios, whereas chickens in the other groups had no different RV:TV ratios.

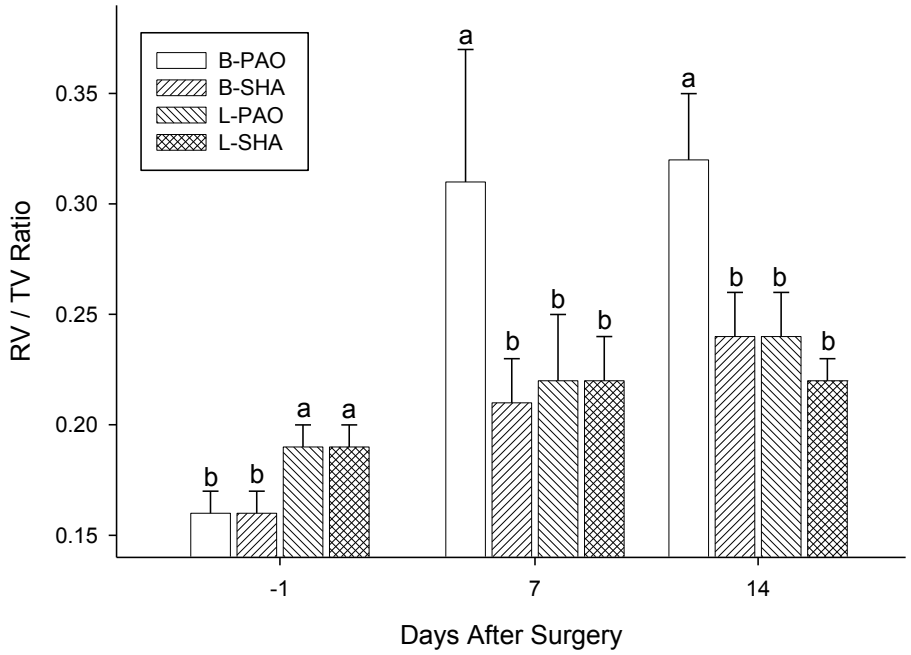


Figure 15. Effect of chicken strain and oxygen level on right ventricle weight to total ventricle weight ratio (RV / TV Ratio). Broiler chickens with one surgically occluded pulmonary artery (B-PAO) and their sham-operated control (B-SHA), and male Leghorns with occluded pulmonary artery (L-PAO) and their control (L-SHA). Data are expressed as means ± SE (n = 8 per group and time point). Means within each sampling point without a common letter are different ($P \leq 0.05$).

There were two cases (2/20; 10 % incidence) of clinical PHS, as evidenced by the presence of ascitic fluid in the abdominal cavity, in the B-PAO chickens, while no clinical cases were seen in the other experimental groups. Before surgery, male Leghorns (average 861 ± 87 g) were heavier than male broiler chickens (average 507 ±

40 g), but at d 7 and 14 post surgery, broilers were heavier than Leghorns. Within strain, there were no differences PAO or SHAM birds (Figure 16).

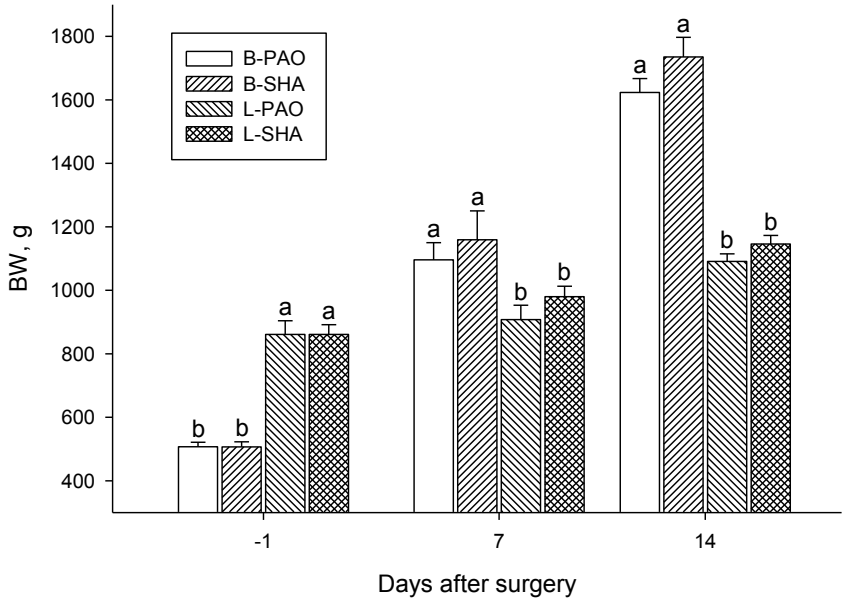


Figure 16. Effect of chicken strain and oxygen level on body weight. Male broiler chickens with one surgically occluded pulmonary artery (B-PAO) and their sham-operated control (B-SHA), and male Leghorns with occluded pulmonary artery (L-PAO) and their control (L-SHA). Data are expressed as means ± SE (n = 8 per group and time point). Means without a common letter are different ($P \leq 0.05$).

DISCUSSION

The present investigation compares physiological responses and resistance pulmonary artery remodeling of male Leghorn, resistant to pulmonary hypertension, and male broiler chickens, susceptible to pulmonary hypertension, after the entire cardiac output is directed to one lung by surgically occluding one pulmonary artery.

Pulmonary Resistance Artery (PRA) Hypertrophy

Small diameter arterioles are responsible for the increased resistance to blood flow in the lungs; for instance, hypoxic vasoconstriction resides proximal to the capillaries and occurs in resistance arterioles 30 - 300 μm in diameter (Reeves and Rubin, 1998). Endothelial dysfunction and pulmonary arterial smooth muscle hypertrophy are key histopathological changes that characterize pulmonary hypertension. Medial thickness (% Thickness) is a reliable estimate of the amount of smooth muscle in several species (Tucker et al., 1975; Moreno de Sandino and Hernandez, 2006). In the present study, we measured medial thickness in two size categories: resistance pulmonary arteries (RPA) of less than 100 μm in diameter, and RPA between 100 and 200 μm in diameter. The reason for this arbitrary differentiation was to better assess the medial thickness response to the PHS-inducing method. For example, it has been shown that PRA < 60 μm diameter show neomuscularization in the peripheral areas of the lung (Xiang et al., 2002). Thus, studying pulmonary arteries < 100 μm separately was deemed to be a better approach to assess responses by previously non-muscularized vessels that become muscularized as a response to PAO. Regarding the smaller arteries (less than 100 μm), broilers and leghorns have non-different arterial thickness before surgery; however B-PAO chickens had thicker RPA (21 ± 0.69 %) than chickens from the other groups at 7 and 14 d post surgery (Figure 11). At d 14 after surgery, B-PAO had thicker PRA than B-SHAM birds, and B-SHAM birds had thicker PRA than leghorns. These results are in agreement with those reporting that broiler chickens with PHS consistently exhibit medial hypertrophy and/or lesions in PRA with diameter less than 100 μm. For example, broiler chickens exposed to suboptimal temperature (12 - 14 °C) presented neomuscularization of pulmonary arteries (< 60 μm in diameter) in the hilum (Pan et al., 2005; Tan et al., 2005). Also, broiler chickens exposed to chronic hypoxia had more lesions in the RPA (50 - 100 μm), evidenced by a reduced nitric oxide synthase expression (source of NO), compared with age-mated birds raised under normoxia (Moreno de Sandino and Hernandez, 2006). Overall, these results

indicate that small diameter pulmonary arteries are more likely to undergo remodeling once the PVR is increased as in the case of PAO used in the present investigation to amplify PHS. Also, the fact that PAO did not lead to increased medial thickness in the L-PAO chickens suggest that Leghorns were capable of avoiding sustained increased PAP endothelial dysfunction.

Larger PRA (100 - 200 μm) were already thicker in broilers than in leghorns before surgery (Figure 12). However, the effects of pulmonary artery occlusion were not consistent on this type of arteries. At d 7, arterial thickness was highest in B-SHAM birds, whereas B-PAO, L-PAO, and L-SHAM birds had similar arterial thickness. Conversely, by d 14 B-PAO birds had thicker PRA than L-PAO birds, but not different from those of B-SHAM or L-SHAM birds. Moreno de Sandino and Hernandez (2006) reported that hypertensive chickens (RV/TV ratio of 0.44 ± 0.03) developed thicker PRA, regardless of diameter (50 - 100 μm, or 100 - 200 μm), compared with non-hypertensive chickens (RV/TV ratio of 0.24 ± 0.03). One reason for this difference may be that in the present investigation all in-experiment hypoxemic broilers (B-PAO) were clinically healthy (RV/TV lower than 0.35), whereas Moreno de Sandino and Hernandez (2006) studied very hypertensive, most probably clinically ascitic broilers. Taken together, these results suggest that the degree of vascular remodeling and the size of the pulmonary artery affected is dependent of the severity of the challenge imposed by the PHS-inducing methods and the degree of hypertension developed by the birds.

Physiological and Morphological Parameters

In the present study broilers had consistently lower relative lung weight, a parameter of ventilation capacity, than Leghorns throughout the experiment (Figure 13). These results agree with those reported by Hassanzadeh et al. (2005), who found that layer Leghorns had a consistently higher lung volume and weight as a percentage of BW than broiler chickens. In their investigation, the authors showed that both parameters were highly correlated from 7 d to 42 d of age. In this regard, a low lung volume reduces

the gas exchange area thus explaining why in broilers the developing cardiopulmonary system fails to keep up with growth rate thus resulting in high susceptibility to PHS, compared to Leghorns under normoxic conditions. Furthermore, this limitation also explains why broilers challenged with factors that increase vascular resistance (e.g. PAO) are more prone to become more hypertensive than their SHAM counterparts in the present investigation.

Unilateral PAO leads to ventilation-perfusion mismatch because the entire cardiac output is forced through the unobstructed pulmonary artery at higher speed with lower time for gas exchange, with a concomitant reduction in arterial PO_2 (Wideman and Kirby, 1995b). In the present investigation, the PO_2 in the blood was not measured but previous research has shown that the PO_2 in the blood drops from 103 mm Hg to 83 mm Hg following acute PAO in anesthetized broiler chickens (Wideman and Kirby, 1995b).

In the present study, L-PAO chickens developed significantly higher polycythemia ($P < 0.05$) than L-SHAM birds at both sampling times following PAO surgery. This result is consistent with the results by Mirsalimi et al. (1993), who reported that male Leghorns fed a high-energy diet (3,075 Kcal / kg feed) and subjected to hypobaric hypoxia (simulated 2054 m altitude) were significantly more polycythemic than normoxic age-mates. Taken together, these results suggest that Leghorns do become polycythemic (not only broilers) suggesting that the response to erythropoietin, released in the circulation as a result of hypoxemia, is maintained across strains of birds. In addition, this result further supports the hypothesis that polycythemia may not be an important factor in PHS in chickens.

The RV/TV ratio has been used as a reliable indicator of pulmonary hypertension (Wideman, 2001). Clinically healthy domestic fowl with normal pulmonary arterial pressure have RV/TV ratios ranging from 0.15 to 0.27, whereas sustained pulmonary hypertension causes RV/TV ratios above 0.28 (Wideman, 2001). In the present study, the higher susceptibility of broiler chickens to pulmonary hypertension compared to Leghorns was evidenced by the fact that B-PAO developed right ventricular hypertrophy

(RV:TV above 0.28 at d 7 and d 14 post surgery) as a result of the sustained pulmonary hypertension, whereas L-PAO chickens did not show signs of sustained hypertension (RV:TV at or below 0.24; Figure 15). These results confirm that broilers have a low capacity pulmonary vasculature, and lower pulmonary vascular vasodilation capacity than Leghorns.

The physiological responses of the experimental birds, in light of our results, can be summarized as follows.

Pulmonary arteries from broilers have a reduced endothelium-dependent relaxation compared to those from Leghorns (Martinez-Lemus et al., 1999; Odom et al., 2004), and that acute hypoxia produces higher constriction of intrapulmonary arteries in broilers than in Leghorns (Zoer et al., 2009). Furthermore, boilers have a low compliance pulmonary vasculature that in PHS-susceptible individuals it is fully engorged with blood at normal cardiac outputs (Wideman, 2001).

In this study leghorns had higher lung capacity than broilers (Figure 13), and this higher ventilation capacity in addition to the morphological and physiological advantages reported above, allowed leghorns to maintain thin PRA even after the PAO, in contrast with the increase in the PRA thickening seen in broilers at 7 d and 14 d post surgery (Figure 11).

Pulmonary artery remodeling, mainly thickening of the media (smooth muscle), is in itself a contributing factor in PHS (Mandegar et al., 2004). In this regard, sustained high pressure (hypertension) in a normally low-pressure pulmonary circulation induces changes in the artery wall including smooth muscle cell proliferation. In the present study, pulmonary artery thickening (especially in resistant arteries < 100 μm; Figure 11) was a contributing factor to PHS that was observed in broiler chickens (B-PAO group). In this regard, increases in PAP elicited thickening of small PRA, but more in broilers than in leghorns, which indicates that arterial thickening is an integral part of PHS development. However, broilers have an age dependent increase of arterial thickening, because at 14 d after surgery, sham operated broilers had thicker arteries than L-PAO or L-SHAM (Figure 11). For example it has been reported that in anesthetized normoxic

broiler chickens, the PAP increases from 18 mm Hg at two wk of age to 25 mm Hg at three wk of age and stays high until 6 wk of age (Forman et al., 2000). Such increases in PAP were attributed to fast growth rate thus placing a burden on the pulmonary vasculature and the right ventricle. This may explain why the broiler chickens showed an age-dependent increase in the RV/TV ratio observed in the present study. Results in the present investigation also showed that, coinciding with the thickening of pulmonary resistance arteries, there was onset of hypertension, measured by an increased RV/TV ratio (Figure 15), again with a higher impact on broiler chickens than in Leghorns. For example, the RV/TV ratio increased 10 % at 14 d post surgery in the L-PAO group relative to the L-SHAM. However, in the B-PAO chickens this parameter increased 48 % and 33 % at 7 d and 14 d post surgery, respectively, relative to the B-SHAM group.

The fact that the RV:TV ratio was similar between the B-SHAM and L-PAO and L-SHAM suggests that increase in RV:TV ratio seen in Leghorns was not physiologicaly relevant and so it may explain why this strain of birds did not develop PHS in the present study.

It is concluded that broiler chickens had a lower ventilation capacity than Leghorns. The fact that both B-PAO and L-PAO became polycythemic but L-PAO neither developed pulmonary hypertension nor showed PA remodeling supports the notion that polycythemia is not a major contributor factor to PHS in chickens.

In broiler chickens, the physiological responses brought about by PAO-induced PVR led to a vicious cycle that included pulmonary hypertension and pulmonary artery remodeling (thickening of resistance pulmonary arteries), where a low pulmonary vascular capacity may have played a more determinant role, rather than hypoxemia. These physiological changes were evidenced by a higher degree of right ventricular hypertrophy and susceptible broilers succumbing to PHS.

However, the slow growth rate of Leghorns may have led to low oxygen demand contributing to resistance to PHS. In addition, since hypoxemia seemed to have played a secondary role in PHS, in Leghorns unlike broilers pulmonary vasculature flow-dependent vasodilation may have played an important role in reducing PVR, pulmonary

artery remodeling and pulmonary hypertension. Finally, the fact that it has been reported elsewhere that broilers exhibit reduced pulmonary artery reactivity to vasodilators under normoxia compared to Leghorns, supports the hypothesis that pulmonary vasodilation helped L-PAO chickens lower their pulmonary arterial pressure.

CHAPTER VII

SUMMARY AND CONCLUSIONS

SUMMARY

The broiler chicken with pulmonary hypertension syndrome (PHS) adequately represents idiopathic pulmonary arterial hypertension (IPAH), a human condition that affects 1-2 people per million in the general population and its cause remains to be identified. According to the Centers for Disease Control the incidence of IPAH may be increasing. The late stages of IPAH are well characterized based on histological studies on lungs from patients succumbing to the condition whereas the triggering factors leading to vascular remodeling and pulmonary hypertension remain elusive.

The onset of PHS and IPAH is associated with an increased production of reactive oxygen species. One of the mechanisms proposed through which oxidative stress is implicated in PHS and IPAH is by reducing the availability of nitric oxide (NO). In this scenario NO reacts with superoxide to produce peroxynitrite reducing vasodilation, increasing vasocontraction, and pulmonary arterial pressure culminating with right ventricular failure.

In the first experiment, it was observed that the chronic oral supplementation with L-arginine (Arg), a substrate for endothelial NO synthase (eNOS), reduced the time that the pulmonary artery took to reach basal levels in hypoxic (induced by hypobaric hypoxia) broiler chickens following two consecutive challenges with epinephrine to induce vasoconstriction. In addition, this response was further improved by the supplementation of vitamin E (VE; lipid-soluble antioxidant vitamin) and vitamin C (VC; water-soluble antioxidant vitamin).

In the second experiment, the same experimental diets and birds from experiment 1 were used to further investigate the source of ROS in the vicinity of the pulmonary artery endothelium. It was demonstrated that NAD(P)H-oxidase (NOX) and xanthine

oxidase (XO), both superoxide-producing enzymes implicated in IPAH, are localized in the vicinity of the pulmonary artery endothelium of hypoxic broiler chickens. More specifically, both enzymes were localized in the cell membrane and within vesicles of endothelial cells. XO activity in the lung parenchyma was the lowest in the hypoxic unsupplemented broilers. Arg-supplemented hypoxic chickens had higher XO activity than the hypoxic unsupplemented ones. In turn, birds supplemented with Arg plus VE and VC had higher XO activity than Arg-supplemented ones. NOX activity and NO availability were not affected by diet or hypoxia. However, hypoxic chickens that developed PHS had higher nitrotyrosine (a marker of oxidative stress and NO availability) than hypoxic clinically healthy ones.

In experiment 3, pulmonary segments from normoxic unsupplemented broilers were less reactive to phenylephrine (PE), an α1-adrenergic agonist, than those from hypoxic unsupplemented ones that had been subjected to unilateral primary bronchus occlusion (PAO) to induce PHS. Also, chronic oral supplementation of Arg or Arg plus VE and VC restored normal reactivity to PE. Supplementation during preincubation with Arg did not affect the maximal response to PE in pulmonary rings from Arg-supplemented birds suggesting that diffusion of Arg limited its availability. Supplementation during preincubation with Arg plus VE and VC reduced the maximal response to PE in pulmonary rings.

In experiment 4, male Leghorns showed consistently higher specific lung weight than male broilers chickens of the same body weight and fed a typical broiler diet. When both strains of chickens were subjected to unilateral pulmonary artery occlusion (PAO), to increase pulmonary vascular resistance to blood flow, broilers were more hypertensive whereas Leghorns were more hypoxemic and less hypertensive. In addition, broilers showed more remodeling in resistance pulmonary arteries < 100 μm in diameter than Leghorns when subjected to PAO. Birds subjected to PAO were more hypoxemic and hypertensive than normoxic ones.

CONCLUSIONS

Chronic supplementation with Arg improves the cardiovascular performance of hypoxic broiler chickens subjected to chronic hypobaric hypoxia. In addition, the cardiovascular performance can be further improved by the chronic supplementation of VE and VC.

Supplemented Arg and VE and VC restore XO activity in and additive manner in hypoxic broilers.

NAD(P)H-oxidase and XO were localized in the vicinity of the pulmonary endothelial cell and supplemented Arg plus VE and VC restores XO activity without affecting NOX activity and oxidative stress. The dual role of XO, which produces superoxide and uric acid (antioxidant), may have a buffering effect on superoxide in clinically healthy birds.

Nitric oxide availability is reduced and oxidative stress is increased in broilers that developed PHS when subjected to chronic hypobaric hypoxia.

Hypoxia increases pulmonary artery reactivity to PE. Chronic supplementation with Arg plus VE and VC restores endothelium-dependent vasorelaxation in hypoxic broiler chickens. Therefore, an increase in reactive oxygen species may be involved in the hyperresponse to PE in the hypoxic pulmonary artery of broiler chickens.

After accounting for sex and body weight, Leghorns have higher ventilation capacity than broilers. Leghorns show a better pulmonary vasodilation capacity than broilers. Thus Leghorns are able to accommodate the entire cardiac output through a single lung avoiding not only an increase in vascular resistance created by PAO but a sustained pulmonary hypertension.

REFERENCES

Abuja, P.M., 1999. Prooxidant effects of uric acid and other aqueous antioxidants are prevented by ascorbic acid. Atherosclerosis. 144, 46-47.

Al-Khalidi, U.A.S. and T.H. Chaglassian. 1965. The species distribution of xanthine oxidase. Biochem. J. 97, 3.

Alp, N. J., and K. M. Channon. 2004. Regulation of endothelial nitric oxide synthase by tetrahydrobiopterin in vascular disease. Arteriosclerosis Thrombosis and Vascular Biology 24:413-420.

Alvarez-Medina, D. I., A. Hernandez, and C. Orozco. 2011. Endothelial hyperpolarizing factor increases acetylcholine-induced vasodilatation in pulmonary hypertensive broilers arterial rings. Res.Vet. Sci. 92:1-6.

Atkinson, J., R. F. Epand, and R. M. Epand. 2008. Tocopherols and tocotrienols in membranes: A critical review. Free Radic. Biol. Med. 44:739-764.

Bautista-Ortega, J. and C.A. Ruiz-Feria. 2010. L-arginine and antioxidant vitamins E and C improve the cardiovascular performance of broiler chickens grown under chronic hypobaric hypoxia. Poult Sci. 89, 2141-2146.

Billaud, M., A. W. Lohman, A. C. Straub, R. Looft-Wilson, S. R. Johnstone et al. 2011. Pannexin1 regulates alpha1-adrenergic receptor- mediated vasoconstriction. Circ. Res. 109:80-85.

Bisby, R. H., and A. W. Parker. 1995. Reaction of ascorbate with the [alpha]-tocopheroxyl radical in micellar and bilayer membrane systems. Arch. Biochem. Biophys. 317:170-178.

Bowers, R., C. Cool, R. C. Murphy, R. M. Tuder, M. W. Hopken et al. 2004. Oxidative stress in severe pulmonary hypertension. Am. J. Respir. Crit. Care Med. 169:764-769.

Burton, R., E. Besch, and A. Smith. 1968. Effect of chronic hypoxia on the pulmonary arterial blood pressure of the chicken. Am. J. Physiol. 214:1438-1442.

Carro, M.D., E. Falkenstein, K.P. Blemings, and H. Klandorf. 2009. Determination of xanthine oxidoreductase activity in broilers: Effect of pH and temperature of the assay and distribution in tissues. Poult Sci. 88, 2406-2414.

Carro, M.D., E. Falkenstein, K.P. Blemings, and H. Klandorf. 2010. Effects of allopurinol on uric acid concentrations, xanthine oxidoreductase activity and oxidative stress in broiler chickens. Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. Part C 151, 6.

Carville, C., B. Raffestin, S. Eddahibi, Y. Blouquit, and S. Adnot. 1993. Loss of endothelium-dependent relaxation in proximal pulmonary arteries from rats exposed to chronic hypoxia: effects of in vivo and in vitro supplementation with L-arginine. J Cardiovasc. Pharmacol. 22:889-896.

Chapman, M. E., and R. F. Wideman. 2006. Evaluation of total plasma nitric oxide concentrations in broilers infused intravenously with sodium nitrite, lipopolysaccharide, aminoguanidine, and sodium nitroprusside. Poult Sci 85:312-320.

Cheng, J., J.S. Ou, H. Singh, J.R. Falck, D. Narsimhaswamy et al. 2008. 20-hydroxyeicosatetraenoic acid causes endothelial dysfunction via eNOS uncoupling. Am. J. Physiol. Heart Circ. Physiol. 294, H1018-H1026.

Clarke, M. W., J. R. Burnett, and K. D. Croft. 2008. Vitamin E in human health and disease. Crit Rev in Clin Lab Sci 45:417 – 450.

Decuypere, E., C. Vega, T. Bartha, J. Buyse, J. Zoons et al. 1994. Increased sensitivity to triiodothyronine (T3) of broiler lines with a high susceptibility for ascites. Brit. Poult. Sci. 35:287-297.

Dietert, R. R., and R. E. Austic. 1994. Environment–immune interactions. Poult.Sci.73:1062–1076.

Dudgeon, S., D. P. Benson, A. MacKenzie, K. Paisley-Zyskiewicz, and W. Martin. 1998. Recovery by ascorbate of impaired nitric oxide-dependent relaxation resulting from oxidant stress in rat aorta. Br. J. Pharmacol. 125:782–786.

Dudzinski, D. M., and T. Michel. 2007. Life history of eNOS: Partners and pathways. Cardiovasc. Res. 75:247-260.

Ellis, E.A. and Grant, M.B., 2002. Cytochemical localization of H_2O_2 in biological tissues. Pages 3-12 in Methods in Molecular Biology, vol. 196: Oxidants and Antioxidants: Ultrastructure and Molecular and Molecular Biology protocols. D. Armstrong, ed. Humana Press Inc., Totowa, NJ.

Enkvetchakul, B., W. Bottje, N. Anthony, R. Moore, and H. Huff. 1993. Compromised antioxidant status associated with ascites in broilers. Poult. Sci. 72:2272-2280.

Fasman, G.D., 1989. Practical Handbook of Biochemistry and Molecular Biology. G.D. Fasman, ed. CRC Press Inc., Boca Raton, FL.

Forman, M., and R. Wideman. 2000. Measurements of pulmonary arterial pressure in anesthetized male broilers at two to seven weeks of age. Poult. Sci. 79:1645-1649.

Goldstein, S., and G. Czapski. 1995. The reaction of $NO^{\cdot}$ with $O2^{\cdot -}$ and $HO2^{\cdot}$: A pulse radiolysis study. Free Radic. Biol. Med. 19:505-510.

Gomez-Arroyo, J. G., L. Farkas, A. A. Alhussaini, D. Farkas, D. Kraskauskas, et al. 2012. The monocrotaline model of pulmonary hypertension in perspective. Am J Physiol Lung Cell Mol Physiol 302:L363-L369.

Govers, R., and T. J. Rabelink. 2001. Cellular regulation of endothelial nitric oxide synthase. Am. J. Physiol. Renal Physiol. 280:F193-206.

Guimarães, S., and D. Moura. 2001. Vascular adrenoceptors: An update. Pharmacol. Rev. 53:319-356.

Guney, M., B. Oral, H. Demirin, N. Karahan, T. Mungan, et al. 2007. Protective effects of vitamins C and E against endometrial damage and oxidative stress in fluoride intoxication. Clin. Exp. Pharmacol. Physiol. 34:467-474.

Guzik, T.J., and K.K. Griendling. 2009. NADPH Oxidases: Molecular Understanding Finally Reaching the Clinical Level? Antiox. Redox Signaling. 11, 2365-2370.

Halliwell, B., and J. M. C. Gutteridge. 1999. Free Radicals in Biology and Medicine. 3rd ed. Oxford University Press, Oxford, England.

Harvey, R.B., L.F. Kubena, S.L. Lovering, and T.D. Philips. 1985. Ketamine/xylazine anesthesia for chickens. Avian Exotic. Pract. 2, 6-7.

Hassanzadeh, M., H. Gilanpour, S. Charkhkar, J. Buyse, and E. Decuypere. 2005. Anatomical parameters of cardiopulmonary system in three different lines of chickens: Further evidence for involvement in ascites syndrome. Avian Pathol. 34, 188-193.

Heller, R., F. Münscher-Paulig, R. Gräbner, and U. Till. 1999. L-Ascorbic acid potentiates nitric oxide synthesis in endothelial cells. J. Biol. Chem. 274:8254-8260.

Houston, M., Estevez, A., Chumley, P., Aslan, M., Marklund, S., et al. 1999. Binding of xanthine oxidase to vascular endothelium - Kinetic characterization and oxidative impairment of nitric oxide-dependent signaling. J. Biol. Chem. 274, 4985-4994.

Kelly, D. A., A. A. Hislop, S. M. Hall, and S. G. Haworth. 2005. Relationship between structural remodeling and reactivity in pulmonary resistance arteries from hypertensive piglets. Pediatr. Res. 58:525-530.

Kim, H. R., S. Appel, S. Vetterkind, S. S. Gangopadhyay, and K. G. Morgan. 2008. Smooth muscle signaling pathways in health and disease. J. Cell. Mol. Med. 12:2165-2180.

Knock, G. A., V. A. Snetkov, Y. Shaifta, M. Connolly, S. Drndarski, et al. 2009. Superoxide constricts rat pulmonary arteries via Rho-kinase-mediated Ca(2+) sensitization. Free Radic. Biol. Med. 46:633-642

Koyama, T., and M. Horimoto. 1983. Blood flow reduction in local pulmonary microvessels during acute hypoxia imposed on a small fraction of the lung. Resp. Physiol. 52, 181-189.

Jandel Scientific. 1994. SigmaStat Statistical Software User's Manual. Jandel Scientific Software, San Rafael, CA.

Jankov, R.P., Kantores, C., Pan, J., Belik, J., 2008. Contribution of xanthine oxidase-derived superoxide to chronic hypoxic pulmonary hypertension in neonatal rats. Am. J. Physiol. Lung Cell Mol. Physiol. 294, L233-245.

Jernigan, N. L., B. R. Walker, and T. C. Resta. 2008. Reactive oxygen species mediate RhoA/Rho kinase-induced Ca(2+) sensitization in pulmonary vascular smooth muscle following chronic hypoxia. Amer. J. Physiol. Lung Cel. Mol. Physiol. 295:L515-L529.

Julian, R.J., 2007. The Response of the Heart and Pulmonary Arteries to Hypoxia, Pressure, and Volume. A Short Review. Poult. Sci. 86, 1006-1011.

Kelley, E.E., T. Hock, N.K.H. Khoo, G.R. Richardson, K.K. Johnson, et al. 2006. Moderate hypoxia induces xanthine oxidoreductase activity in arterial endothelial cells. Free Radical Biol. Med. 40, 952-959.

Kojda, G., and D. Harrison. 1999. Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. Cardiovasc. Res. 43:562-571.

Koyama, T., and M. Horimoto. 1983. Blood flow reduction in local pulmonary microvessels during acute hypoxia imposed on a small fraction of the lung. Respiration Physiology 52:181-189.

Lassegue, B., and K.K. Griendling. 2010. NADPH Oxidases: Functions and Pathologies in the Vasculature. Arterioscler., Thromb., Vasc. Biol. 30, 653-661.

Liu, J.F., and Y.W. Lee. 1998. Vitamin C supplementation restores the impaired vitamin E status of guinea pigs fed oxidized frying oil. J. Nutr. 128:116-122.

Lorenzoni, A. G., and C. A. Ruiz-Feria. 2006. Effects of vitamin E and L-arginine on cardiopulmonary function and ascites parameters in broiler chickens reared under subnormal temperatures. Poult. Sci. 85:2241-2250.

Luscher, T., Z. Yang, M. Tschudi, L. von Segesser, P. Stulz, et al. 1990. Interaction between endothelin-1 and endothelium-derived relaxing factor in human arteries and veins. Circ. Res. 66:1088-1094.

Mandegar, M.,Y-C. B. Fung, W. Huang, C. V. Remillard, L. J. Rubin, et al. 2004. Cellular and molecular mechanisms of pulmonary vascular remodeling: role in the development of pulmonary hypertension. Microvasc. Res. 68, 75-103.

Martinez-Lemus, L.A., R.K. Hester, E.J. Becker, J.S. Jeffrey and T.W. Odom. 1999. Pulmonary artery endothelium-dependent vasodilation is impaired in a chicken model of pulmonary hypertension, Am. J. Physiol. Regul. Integr. Comp. Physiol. R190-197.

Mishra, N., W. H. Nugent, S. Mahavadi, and S. W. Walsh. 2011. Mechanisms of Enhanced Vascular Reactivity in Preeclampsia. Hypertension. 58:867-873.

Moreno de Sandino, M., and A. Hernandez. 2006. Pulmonary arteriole remodeling in hypoxic broilers expressing different amounts of endothelial nitric oxide synthase. Poult. Sci. 85, 899-901.

Maxwell, M.H., Robertson, G.W., Farquharson, C., 1996. Evidence of untrcytochemical mitochondria-derived hydrogen peroxide activity in myocardial cells from broiler chickens with an ascites syndrome. Res.Vet. Sci. 61: 7-12.

McLaughlin, V. V., S. L. Archer, D. B. Badesch, R. J. Barst, H. W. Farber, et al. 2009. ACCF/AHA 2009 Expert Consensus Document on Pulmonary Hypertension: A Report of the American College of Cardiology Foundation Task Force on Expert Consensus Documents and the American Heart Association developed in collaboration with the American College of Chest Physicians; American Thoracic Society, Inc.; and the Pulmonary Hypertension Association. J. Am. Coll. Cardiol. 53:1573-1619.

McLaughlin, V. V., M. Davis, and W. Cornwell. 2011. Pulmonary Arterial Hypertension. Current Problems in Cardiology 36:461-517.

McNally, J.S., M.E. Davis, D.P. Giddens, A. Saha, J. Hwang, et al. 2003. Role of xanthine oxidoreductase and NAD(P)H oxidase in endothelial superoxide production in response to oscillatory shear stress. Am. J. Physiol. Heart Circ. Physiol. 285, H2290-2297.

Moreno de Sandino, M., and A. Hernandez. 2003. Nitric Oxide Synthase Expression in the Endothelium of Pulmonary Arterioles in Normal and Pulmonary Hypertensive Chickens Subjected to Chronic Hypobaric Hypoxia. Avian Diseases 47:1291-1297.

Moreno de Sandino, M., and A. Hernandez. 2006. Pulmonary arteriole remodeling in hypoxic broilers expressing different amounts of endothelial nitric oxide synthase. Poult Sci 85:899-901.

Nagaoka, S.i., T. Kakiuchi, K. Ohara, and K. Mukai. 2007. Kinetics of the reaction by which natural vitamin E is regenerated by vitamin C. Chem. Phys. Lipids 146:26-32.

Nagasaka, Y., J. Bhattacharya, S. Nanjo, M.A. Cropper, and N.C. Staub. 1984. Micropuncture Measurement of Lung Microvascular Pressure Profile during Hypoxia in Cats. Circ. Res. 54, 90-95.

Nain, S., B. Ling, B. Bandy, J. Alcorn, C. Wojnarowicz, B. Laarveld, and A. A. Olkowski. 2008a. The role of oxidative stress in the development of congestive heart failure in a chicken genotype selected for rapid growth. Avian Pathol. 37:367-373.

Nain, S., C. Wojnarowicz, B. Laarveld, and A. A. Olkowski. 2008b. Effects of dietary vitamin E and C supplementation on heart failure in fast growing commercial broiler chickens. Br. Poult. Sci. 49:697-704.

National Research Council. 1994. Nutrient Requirements of Poultry. 9th rev. ed. Natl. Acad. Press, Washington, DC.

Odom, T. W., L. A. Martinez-Lemus, R. K. Hester, E. J. Becker, J. S. Jeffrey, et al. 2004. In vitro hypoxia differentially affects constriction and relaxation responses of isolated pulmonary arteries from broiler and leghorn chickens. Poult. Sci. 83:835-841.

Ohkawa, H., N. Ohishi, and K. Yagi. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351-358.

Olson, S. E., and G. E. Seidel. 2000. Culture of in vitro-produced bovine embryos with vitamin E improves development in vitro and after transfer to recipients. Biol. Reprod. 62:248-252.

Pacher, P., A. Nivorozhkin, and C. Szabo. 2006. Therapeutic effects of xanthine oxidase inhibitors: renaissance half a century after the discovery of allopurinol. Pharmacol. Rev. 58, 87-114.

Pan, J.Q., X. Tan, J.C. Li, W.D. Sun, and X.L. Wang. 2005. Effects of early feed restriction and cold temperature on lipid peroxidation, pulmonary vascular remodelling and ascites morbidity in broilers under normal and cold temperature. Brit. Poult. Sci. 46, 374-381.

Pan, J. Q., J. C. Li, X. Tan, W. D. Sun, J. Y. Wang et al. 2007. The injury effect of oxygen free radicals in vitro on cultured pulmonary artery endothelial cells from broilers. Res. Vet. Sci. 82:382-387.

Pardue, S. L., J. P. Thaxton, and J. Brake. 1984. Plasma ascorbic acid concentration following ascorbic acid loading in chicks. Poult. Sci. 63:2492-2496.

Poss, W.B., T.P Huecksteadt, P.C Panus, B.A. Freeman, J. R. Hoidal. 1996. Regulation of xanthine dehydrogenase and xanthine oxidase activity by hypoxia. Am. J. Physiol. 270, L941-946.

Rabinovitch, M. 2007. Pathobiology of pulmonary hypertension. Annu. Rev. Pathol. Mech. Dis. 2:369-399.

Ramamurthi, A., and R. S. Lewis. 1997. Measurement and modeling of nitric oxide release rates for nitric oxide donors. Chemical Research in Toxicology 10:408-413.

Reeves, J.T., and L.J. Rubin. 1998. The Pulmonary Circulation . Snapshots of Progress. Am. J. Respir. Crit. Care Med. 157, S101-108.

Robinson, J.M. and B.E. Batten. 1990. Localization of cerium-based reaction products by scanning laser reflectance confocal microscopy. J. Histochem. Cytochem. 38, 315-318.

Rossig, L., J. Hoffmann, B. Hugel, Z. Mallat, A. Haase, et al. 2001. Vitamin C inhibits endothelial cell apoptosis in congestive heart failure. Circulation 104:2182-2187.

Rubbo, H., R. Radi, D. Anselmi, M. Kirk, S. Barnes, et al. 2000. Nitric Oxide Reaction with Lipid Peroxyl Radicals Spares Œ±-Tocopherol during Lipid Peroxidation. J. Biol. Chem. 275:10812-10818.

Rubino, A., and G. Burnstock. 1994. Recovery after dietary vitamin E supplementation of impaired endothelial function in vitamin E-deficient rats. Br. J. Pharmacol. 112:515-518.

Ruiz-Feria, C. A. 2009. Concurrent supplementation of arginine, vitamin E, and vitamin C improve cardiopulmonary performance in broilers chickens. Poult. Sci. 88:526-535.

Ruiz-Feria, C., K. Beers, M. Kidd, and R. Wideman. 1999. Plasma taurine levels in broilers with pulmonary hypertension syndrome induced by unilateral pulmonary artery occlusion. Poult. Sci. 78:1627-1633.

Ruiz-Feria, C. A., M. T. Kidd, and R. F. Wideman. 2001. Plasma levels of arginine, ornithine, and urea and growth performance of broilers fed supplemental L-arginine during cool temperature exposure. Poult. Sci. 80:358-369.

Russell, M. J., R. A. Dombkowski, and K. R. Olson. 2008. Effects of hypoxia on vertebrate blood vessels. Journal of Experimental Zoology Part A: Ecol. Genet. Physiol. 309A:55-63.

Selemidis, S., C.G. Sobey, K. Wingler, H.H.H.W. Schmidt, and G.R. Drummond. 2008. NADPH oxidases in the vasculature: Molecular features, roles in disease and pharmacological inhibition. Pharmacol. Ther. 120, 254-291.

Serbecic, N., and S. C. Beutelspacher. 2005. Anti-oxidative vitamins prevent lipid peroxidation and apoptosis in corneal endothelial cells. Cell Tissue Res. 320:465-475.

Shimoda, L. A., J. S. Sham, and J. T. Sylvester. 2000. Altered pulmonary vasoreactivity in the chronically hypoxic lung. Physiol. Res. 49:549-560.

Shuvaev, V. V., M. Christofidou-Solomidou, F. Bhora, K. Laude, H. Cai, et al. (2009). Targeted detoxification of selected reactive oxygen species in the vascular endothelium. J. Pharmacol. Exp. Therap. **331**(2): 404-411.

Simonneau, G., I. M. Robbins, M. Beghetti, R. N. Channick, M. Delcroix, et al. 2009. Updated Clinical Classification of Pulmonary Hypertension. Journal of the American College of Cardiology 54:S43-S54.

Stallone, J. N. 1994. Sex differences in nitric oxide-mediated attenuation of vascular reactivity to vasopressin are abolished by gonadectomy. European J. Pharmacol. 259:273-283.

Stenmark, K. R., N. J. Davie, J. T. Reeves, and M. G. Frid. 2005. Hypoxia, leukocytes, and the pulmonary circulation. J Appl Physiol 98:715-721.

Stenmark, K. R., B. Meyrick, N. Galie, W. J. Mooi, and I. F. McMurtry. 2009. Animal models of pulmonary arterial hypertension-The hope for etiologic discovery and pharmacologic cure. Am. J. Physiol. Lung Cell. Mol. Physiol. 297:L1013-1032.

Stinefelt, B., S.S. Leonard, K.P. Blemings, X. Shi, and H. Klandorf. 2005. Free Radical Scavenging, DNA Protection, and Inhibition of Lipid Peroxidation Mediated by Uric Acid. Ann. Clin. Lab. Sci. 35, 37-45.

Sud, N., S. Sharma, D.A. Wiseman, C. Harmon, S. Kumar, et al. 2007. Nitric oxide and superoxide generation from endothelial NOS: modulation by HSP90. Am. J. Physiol. Lung Cell Mol. Physiol. 293, L1444-1453.

Surai, P. F. 2006. Antioxidant systems in animal body. Pages 974 in Selenium in Nutrition and Health. N. U. Press ed., Trowbridge, UK.

Tan, X., J.Q. Pan, J.C. Li, Y.J. Liu, W.D. Sun, et al. 2005. L-Arginine inhibiting pulmonary vascular remodelling is associated with promotion of apoptosis in pulmonary arterioles smooth muscle cells in broilers. Res. Vet. Sci. 79:203-209.

Tan, X., H. Song-Hua, and W. Xiao-Long. 2007a. Possible role of nitric oxide in the pathogenesis of pulmonary hypertension in broilers: a synopsis. Avian Pathol. 36:261-267.

Tan, X., W.D. Sun, J.C. Li, J.Q. Pan, Y.J. Liu, et al. 2007b. L-Arginine prevents reduced expression of endothelial nitric oxide synthase (NOS) in pulmonary arterioles of broilers exposed to cool temperatures. Vet. J. 173:151–157.

Terada, L.S., 2006. Specificity in reactive oxidant signaling: think globally, act locally. J. Cell Biol. 174, 615-623.

Thomas, D.D., L.A. Ridnour, M.G. Espey, S. Donzelli, S. Ambs, et al. 2006. Superoxide fluxes limit nitric oxide-induced signaling. J. Biol. Chem. 281, 25984-25993.

Thomas, S. R., E. Schulz, and J. F. Keanney. 2006. Hydrogen peroxide restrains endothelium-derived nitric oxide bioactivity- Role for iron-dependent oxidative stress. Free Radic. Biol. Med. 41:8.

Tucker, A., I. McMurtry, J. Reeves, A. Alexander, D. Will, et al. 1975. Lung vascular smooth muscle as a determinant of pulmonary hypertension at high altitude. Am. J. Physiol. 228, 762-767.

Tuder, R. M., S. H. Abman, T. Braun, F. Capron, T. Stevens, et al. 2009. Development and Pathology of Pulmonary Hypertension. Journal of the American College of Cardiology 54:S3-S9.

Tulloh, R. M., A. A. Hislop, P. J. Boels, J. Deutsch, and S. G. Haworth. 1997. Chronic hypoxia inhibits postnatal maturation of porcine intrapulmonary artery relaxation. Am. J Physiol. 272:H2436-2445.

van der Vliet, A., 2008. NADPH oxidases in lung biology and pathology: host defense enzymes, and more. Free Radic. Biol. Med. 44, 938-955.

Vincent, A.M., L. Perrone, K.A. Sullivan, C. Backus, A.M. Sastry, C. Lastoskie, et al. 2007. Receptor for Advanced Glycation End Products Activation Injures Primary Sensory Neurons via Oxidative Stress. Endocrinology. 148, 548-558.

Walshe, T. E., and P. A. D'Amore. 2008. The role of hypoxia in vascular injury and repair. Annu Rev Pathol: Mechanisms of Disease 3:615-643.

Ward, J. P. T., and G. A. Knock. 2011. Excitation–contraction coupling and regulation of pulmonary vascular contractility. Pages 147-165 in Textbook of Pulmonary

Vascular Disease. J. X. J. Yuan, J. G. N. Garcia, J. B. West, C. A. Hales, S. Rich, and S. L. Archer, eds. Springer, New York, NY.

Warwick, G., P.S. Thomas, and D.H. Yates. 2008. Biomarkers in pulmonary hypertension. Eur. Respir. J. 32, 503-512.

Wedgwood, S., and S. M. Black. 2003. Role of reactive oxygen species in vascular remodeling associated with pulmonary hypertension. Antioxid. Redox Signal. 5:759-769.

Wideman, R.F., 2001. Pathophysiology of heart/lung disorders: pulmonary hypertension syndrome in broiler chickens. Worlds Poult. Sci. J. 57, 289-307.

Wideman, R. F., M. E. Chapman, and G. F. Erf. 2005. Pulmonary and systemic hemodynamic responses to intravenous prostacyclin in broilers. Poult. Sci. 84:442-453.

Wideman, R. F., M. E. Chapman, C. M. Owens, M. K. Devabhaktuni, L. C. Cavitt, W. et al. 2003. Broiler survivors of intravenous micro-particle injections: Evaluation of growth, livability, meat quality, and arterial blood gas values during a cyclic heat challenge. Poult. Sci. 82:484-495.

Wideman, R. F., and G. F. Erf. 2002. Intravenous micro-particle injection and pulmonary hypertension in broiler chickens: Cardio-pulmonary hemodynamic responses. Poult. Sci. 81:877-886.

Wideman, R. F., G. F. Erf, M. E. Chapman, W. Wang, N. B. Anthony et al. 2002. Intravenous micro-particle injections and pulmonary hypertension in broiler chickens: Acute post-injection mortality and ascites susceptibility. Poul. Sci. 81:1203-1217.

Wideman, R.F., M. Forman, J. Hughes, Y. K. Kirby, N. Marson et al. 1998. Flow-dependent pulmonary vasodilation during acute unilateral pulmonary artery occlusion in Jungle Fowl. Poult .Sci. 77, 615-626.

Wideman, R. F. and H. French. 1999. Broiler breeder survivors of chronic unilateral pulmonary artery occlusion produce progeny resistant to pulmonary hypertension syndrome (ascites) induced by cool temperatures. Poult Sci. 78:404-411.

Wideman, R. F., and K. R. Hamal. 2011. Idiopathic pulmonary arterial hypertension: An avian model for plexogenic arteriopathy and serotonergic vasoconstriction. Journal of Pharmacological and Toxicological Methods 63:283-295.

Wideman, R. F., K. R. Hamal, M. T. Bayona, A. G. Lorenzoni, D. Cross et al. 2011. Plexiform lesions in the lungs of domestic fowl selected for susceptibility to pulmonary arterial hypertension: incidence and histology. Anat. Rec. 294:739-755.

Wideman, R.F., and Y.K. Kirby. 1995a. A pulmonary-artery clamp model for inducing pulmonary-hypertension syndrome (ascites) in broilers. Poult. Sci. 74, 805-812.

Wideman, R.F., and Y.K. Kirby. 1995b. Evidence of a ventilation-perfusion mismatch during acute unilateral pulmonary-artery occlusion in broilers. Poult. Sci. 74, 1209-1217.

Wideman, R. F., Y. K. Kirby, C. D. Tackett, N. E. Marson, and R. W. McNew. 1996. Cardio-pulmonary function during acute unilateral occlusion of the pulmonary artery in broilers fed diets containing normal or high levels of arginine-HCl. Poult. Sci. 75:1587-1602.

Wideman, R.F., Kirby, Y.K., Ismail, M., Bottje, W.G., Moore, R.W. et al. (1995). Supplemental L-arginine attenuates pulmonary hypertension syndrome (ascites) in broilers. Poult Sci. 74:323-330.

Wrutniak-Cabello, C., F. Casas, and G. Cabello. 2001. Thyroid hormone action in mitochondria. J. Mol. Endocrinol. 26:67-77.

Xiang, R.P., W.D. Sun, J.Y. Wang, and X.L. Wang. 2002. Effect of Vitamin C on pulmonary hypertension and muscularisation of pulmonary arterioles in broilers, Brit. Poult. Sci. 43, 705-712.

Yeo, W.S., S.J. Lee, J.R. Lee, and K.P. Kim. 2008. Nitrosative protein tyrosine modifications: biochemistry and functional significance. BMB Rep. 41, 194-203.

Zalba, G., Beaumont, F.J., Jose, G.S., Fortuno, A., Fortuno et al. 2000. Vascular NADH/NADPH oxidase is involved in enhanced superoxide production in spontaneously hypertensive rats. Hypertension. 35, 1055-1061.

Zoer, B., L. Kessels, A. Vereijken, J. G. R. De Mey, V. Bruggenman, et al. 2009. Effects of prenatal hypoxia on pulmonary vascular reactivity in chickens prone to pulmonary hypertension. J. Physiol. Pharmacol. 60:119-130.

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